DEVELOPMENTAL TOXICITY OF POLYBROMINATED DIPHENYLETHERS AND POLYCHLORINATED BIPHENYLS: ESTROGEN HORMONE-REGULATED GENE EXPRESSION IN SEXUALLY DIMORPHIC RAT BRAIN REGIONS AND FEMALE SEXUAL BEHAVIOR

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„Die Diskussion darüber, welchen Anteil an der Entwicklung des Verhaltens von Tieren genetischen Ursprungs ist und welchen Anteil daran die Umwelt hat ist genauso hilfreich wie die Diskussion, welchen Anteil am Erklingen einer Klaviersonate der Pianist und welchen das Instrument hat. Das Klavier kann noch so gut gestimmt und noch so sorgfältig gezimmert sein, ohne die künstlerischen Fähigkeiten des Pianisten würde kein einziger Ton erklingen. Ebenso kann der Pianist noch so begabt sein, ohne ein Instrument, das ihm zur Entfaltung zur Verfügung steht, könnte auch er keine einzige Note zum Erklingen bringen. Nur das Zusammenspiel beider Seiten erlaubt uns den Genuss wunderschöner Musik. Genauso verhält es sich mit den Erbanlagen und der Umwelt. Die Gene stellen gewissermaßen die Klaviatur dar, auf der die Natur ihre Entfaltung findet. Nur im Zusammenspiel beider ergibt sich die Vielfalt der Organismen, die uns immer wieder staunend in Ehrfurcht versetzt.“

Konrad Lorenz (1903 – 1989), zitiert in “Das Verhalten von Hunden”, Heidenberger, E., Augustus-Verlag München, 2000

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Summary

For quite some time the possible ability of chemicals to interfere with developmental processes in all kinds of organisms became a matter of concern. Their possible interaction with the endocrine system and therefore their potential to disrupt proper development makes chemicals a target of research since their global occurrence might put species at risk on a global scale. In fact the extinction of the European otter (*Lutra lutra*) in Switzerland is suspected to be due to the contamination of its major food source (fish) by endocrine disrupting chemicals (EDC), as stated in a 1999 report released by the Swiss Agency for Environment, Forests and Landscape (BUWAL). Thus further research on the impact of potential EDC on developmental processes in mammals is an important issue.

In early stages sex-hormones regulate the sexual development of an organism. Therefore ontogeny is a potential target for EDC. In mammals, sexual differentiation of the brain is driven by estradiol, which is derived from testosterone, catalyzed by the enzyme aromatase.

Due to its structural similarity to the well known endocrine disrupters PCB (polychlorinated biphenyls) the brominated flame retardant PBDE99 (pentabromodiphenyl ether congener 99) has been chosen to test its developmental toxicity in rats, with special emphasis on sexual development of the brain. To evaluate the results of PBDE99 and to assess the reproducibility of well known results with PCB under the conditions of this study an industrial PCB-mixture (Aroclor1254) has been chosen as positive control.

In the current study, the possible effects on estrogen-regulated genes were analyzed by measuring mRNA levels in two sexually dimorphic brain regions, the medial preoptic area (MPO) and the ventromedial hypothalamus (VMH) of adult rat offspring, after developmental exposure to PBDE99. Therefore the test-compound was administered by subcutaneous injections to pregnant rats during sensitive phases of brain development in two doses of 1 and 10 mg/kg/day. Since the chemicals are lipophilic they accumulate in fat tissue and therefore exposure of offspring continues through pregnancy, lactation and adulthood. The study comprises two different experimental designs. First, the animals were exposed to PBDE99 until adulthood without any further treatment (baseline experiment). Then, to analyze the sensitivity to estradiol, another series of PBDE99-exposed offspring were gonadectomized at

the age of 10 weeks. After a recovery-period of two weeks, they were subcutaneously injected with one dose of estradiol and sacrificed six hours later to remove the brains (estrogen challenge).
The respective brain regions were cut out from frozen brain slices. The mRNA encoding for estrogen receptor alpha (ER alpha), estrogen receptor beta (ER beta), progesterone receptor (PR) and preproenkephalin (PPE) were determined by Real-time-PCR, using the standard curve method. Cyclophilin served as reference gene.
The results of this study indicate that developmental exposure to PBDE99 affects mRNA expression of estrogen-regulated genes in adult rat offspring in a region- and sex specific manner. In the baseline experiment, treatment with PBDE99 leads to an abolishment of the original sex difference in PR mRNA levels in the VMH of untreated controls. Since PR mRNA level was found to be directly linked to female sexual behavior (Ogawa et al., 1994) treated females were tested with regard to this. Indeed a significant reduce in the performance of female sexual behavior could be detected in this study. In the acute estrogen challenge the effect of PBDE99 on the response to estrogen in gonadectomized adult offspring is gene dependent, and differs between sexes and brain regions. It turned out that the results obtained by treatment with the PCB-mixture Aroclor1254 differed from those obtained by treatment with the test compound PBDE99 in both parts of the study, in the baseline experiment and in the estrogen challenge.
The present study reveals that developmental exposure to PBDE99, an endocrine active flame retardant, can affect sexual brain differentiation processes at the molecular level. The effects occurred at dosages that did not show any signs of general, non-specific toxicity. The alterations found may potentially interfere with the reproductive fitness of populations and are therefore a matter of concern as to the possible impact of the tested compounds on the environment.
The analysis of gene expression represents a novel approach in research on hormonal effects of possible EDC on brain function. The results of this study reveal that hormone-regulated genes in the brain are a potential target of EDC. Hence, the molecular analysis provides a potent tool for the detection of these chemicals.

Zusammenfassung

Die Fähigkeit von Chemikalien in der Umwelt mit dem Hormonsystem sich entwickelnder Organismen zu interferieren gibt seit geraumer Zeit Anlass zur Sorge. Ihr möglicher Eingriff in das Hormonsystem und daher ihr Potential die Entwicklung von Organismen zu stören lässt sie von zunehmendem Interesse für die Forschung werden. Ihr weltweites Vorkommen könnte zu einem globalen Risiko für das Überleben von Arten werden. Tatsächlich wird das Aussterben des Europäischen Fischotters (*Lutra lutra*) in der Schweiz in einem Bericht des Bundesamtes für Umwelt, Wald und Landschaft (BUWAL) von 1999 in Verbindung gebracht mit der Kontamination seiner Hauptnahrungsquelle (Fisch) mit hormonaktiven Chemikalien (endocrine disrupting chemicals = EDC). Daher ist die Forschung über den Einfluss von EDC auf Entwicklungsprozesse bei Säugetieren ein wichtiges Anliegen.

In frühen Entwicklungsstadien regulieren Sexualhormone die sexuelle Entwicklung eines Organismus. Deshalb ist die Ontogenie ein mögliches Ziel für den Eingriff von EDC in diesen Prozess. Bei Säugetieren wird die sexuelle Differenzierung des Gehirns durch Estradiol gefördert. Dieses wird durch das Enzym Aromatase aus Testosteron gebildet.

Wegen seiner ähnlichen chemischen Struktur mit den bekannten hormonaktiven PCBs (Polychlorierte Biphenyle) wurde das bromierte Flammschutzmittel PBDE99 (Polybromierter Diphenylether Kongener 99) ausgewählt, um es auf seine potentielle Entwicklungstoxizität in Ratten zu testen. Dabei lag der Schwerpunkt auf der sexuellen Differenzierung des Gehirns. Um sowohl die Ergebnisse von PBDE99 als auch die Vergeichbarkeit mit wohlbekannten Resultaten bei PCBs unter den Bedingungen dieser Studie bewerten zu können, wurde eine industrielle PCB-Mischung (Aroclor1254) als Positivkontrolle ausgewählt.

In der vorliegenden Studie wurden die möglichen Auswirkungen auf estrogenregulierte Gene analysiert durch die Messung von mRNA-Mengen in zwei sexuell dimorphen Gehirnregionen, der medialen präoptischen Region (MPO) und dem ventromedialen Hypothalamus (VMH) nach Exposition durch PBDE99 während der Entwicklung. Dafür wurde die Testchemikalie in zwei Dosen (1 bzw. 10 mg/kg/Tag) trächtigen Ratten während sensibler Phasen in der Entwicklung des Gehirns durch subkutane Injektionen verabreicht. Wegen ihrer lipophilen Eigenschaften akkumulieren sie im Fettgewebe, und daher dauert die Exposition fort von der Trächtigkeit über die Säugephase bis in die Adultzeit. Die Studie umfasst

zwei unterschiedliche experimentelle Teile. Im ersten Teil wurden die Tiere lediglich mit PBDE99 behandelt ohne weitere Manipulation (baseline). Im zweiten Teil wurden, um die Sensitivität gegenüber Estradiol zu testen, 10 Wochen alte PBDE99-behandelte Junge im Alter von 10 Wochen gonadektomiert. Nach einer Erholungsphase von zwei Wochen, bekamen sie ein s.c. Behandlung mit einer Dosis Estradiol. Sechs Stunden später wurden sie getötet und die Gehirne entnommen (estrogen challange).
Die jeweiligen Gehirnregionen wurden aus gefrorenen Gehirnschnitten herausgestanzt. Die mRNA, die für die Gene Estrogenrezeptor alpha, Estrogenrezeptor beta, Progesteronrezeptor und Preproenkephalin kodieren, wurden mittels realtime-PCR bestimmt. Dabei wurde die Standardkurvenmethode angewandt und Cyclophillin als Referenzgen genutzt.
Die Ergebnisse dieser Studie deuten darauf hin, dass die Behandlung mit PBDE99 während der Entwicklung die Expression von mRNA estrogenregulierter Gene verändert. Dies geschieht in regionen- und geschlecht-spezifischer Weise. Im baseline-Experiment verschwand der Unterschied im PR-mRNA-level zwischen Weibchen und Männchen, der bei unbehandelten Kontrollen sichtbar wurde. Da Ogawa et al., 1994 gezeigt haben, dass die mRNA-Menge an PR im VMH direkt in Verbindung gebracht werden kann mit dem Rückgang von weiblichem Sexualverhalten, wurden die behandelten Weibchen untersucht im Hinblick darauf. Und tatsächlich wurde in dieser Studie ein signifikanter Rückgang in der Zurschaustellung weiblichen Sexualverhaltens beobachtet. Im Akutstudien-Teil war der Effekt der Behandlung adulter gonadektomierter Nachkommen mit PBDE99 auf die Antwort auf Estrogene genabhängig und variierte zwischen den Geschlechtern und den verschiedenen Gehirnregionen. Es stellte sich heraus, dass in beiden Teilen der Studie (sowohl im baseline-Experiment, als auch im Akutversuch) der Einfluss von PBDE99 sich von dem der PCB-Mischung deutlich unterschied.
Mit der vorliegenden Studie konnte gezeigt werden, dass die Exposition gegenüber dem hormonaktiven Flammschutzmittel PBDE99 während der Entwicklung die sexuelle Differenzierung auf molekularer Ebene beeinflussen kann. Die Effekte traten bei Dosierungen auf, die keine Zeichen allgemeiner, unspezifischer Toxizität zur Folge hatten. Die beobachteten Veränderungen sind möglicherweise geeignet, die biologische Fitness von Organismen zu beeinträchtigen und geben deshalb Anlass zur Sorge im Hinblick auf den Einfluss der getesteten Substanzen auf die Umwelt.

Die Analyse von Genexpressionsdaten stellt einen neuen Ansatz zur Untersuchung hormoneller Wirkungen möglicher EDC auf die Funktion des Gehirns dar. Die Ergebnisse dieser Studie zeigen, dass hormonregulierte Gene im Gehirn einen möglichen Angriffspunkt für die Wirkung von EDC sein können. Dadurch bietet die molekulare Analyse ein geeignetes Werkzeug für die Aufdeckung der hormonellen Wirkung dieser Chemikalien.

INTRODUCTION

Development and environment

The reciprocal impact of environment vs. genetics on the development of organisms is a matter of long-lasting debates. It will not be solved finally in this study as well, but the results of this study will clearly show, that the chemically contaminated environment on our planet may have a severe influence on mammalian development. The following introduction serves to give an overview on the state of the art in sexual development in mammals and from there it will become clear how endocrine disrupting chemicals interfere with this process.

Sexual development in mammals

Determination of sex

In birds and mammals, the sexual phenotype depends on the combination of heterosomes, like X and Y in mammals: XX means female, XY male. The presence of a Y chromosome determines the differentiation of the bipotential mammalian gonads into testes. Within this differentiation process the SRY gene (sex determining region on the Y chromosome) plays a crucial part. Once SRY is activated the developmental pathway to testis formation is initiated. The whole complicated process involves a set of genes, among them SF1 (steroidogenic factor 1), which initialises the synthesis of testosterone in the Leydig cells as well as the secretion of the anti Mullerian hormone (AMH) in the Sertoli cells. This secretion in turn leads to the regression of the Mullerian duct and to the formation of the epidydimis, seminal vesicles and the vas deferens.

Testosterone is the differentiation signal for the inner male genital organs. In the prostate, a urogenital sinus derivative, testosterone is converted into 5α-dihydrotesto-sterone (DHT), which shows higher affinity to androgen receptors. Furthermore, testosterone is converted into estradiol by the enzyme aromatase. Since prostate development depends on a combination of androgenic and estrogenic actions, both hormones are necessary for proper urogenital development.

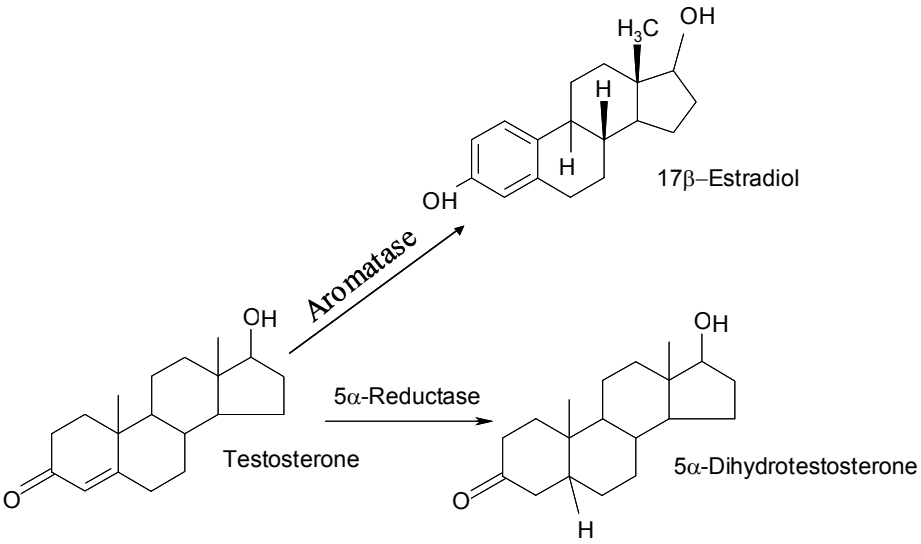


Fig. 1: Aromatase action to convert testosterone to estradiol or 5α-reductase action to convert it to dihydrotestosterone

If the organism carries two X-chromosomes, the gonadal development takes the female route, forming the ovaries. DAX1, a gene on the X chromosome, is expressed in the same cells as SRY and suppresses SF1 expression. The autosomal gene WNT4, expressed in XX gonads, determines ovary development. Under its influence the mesenchym cells of the ovary differentiate into the thecal cells. Together thecal and granulosa cells form the follicles, which secrete steroid hormones. The inhibition of SF1 by DAX1 in turn inhibits the expression of AMH leading to persistence of the Mullerian duct, which then differentiates into the oviducts, uterus, cervix and upper vagina. The Wolffian duct degenerates (Gilbert 2000).

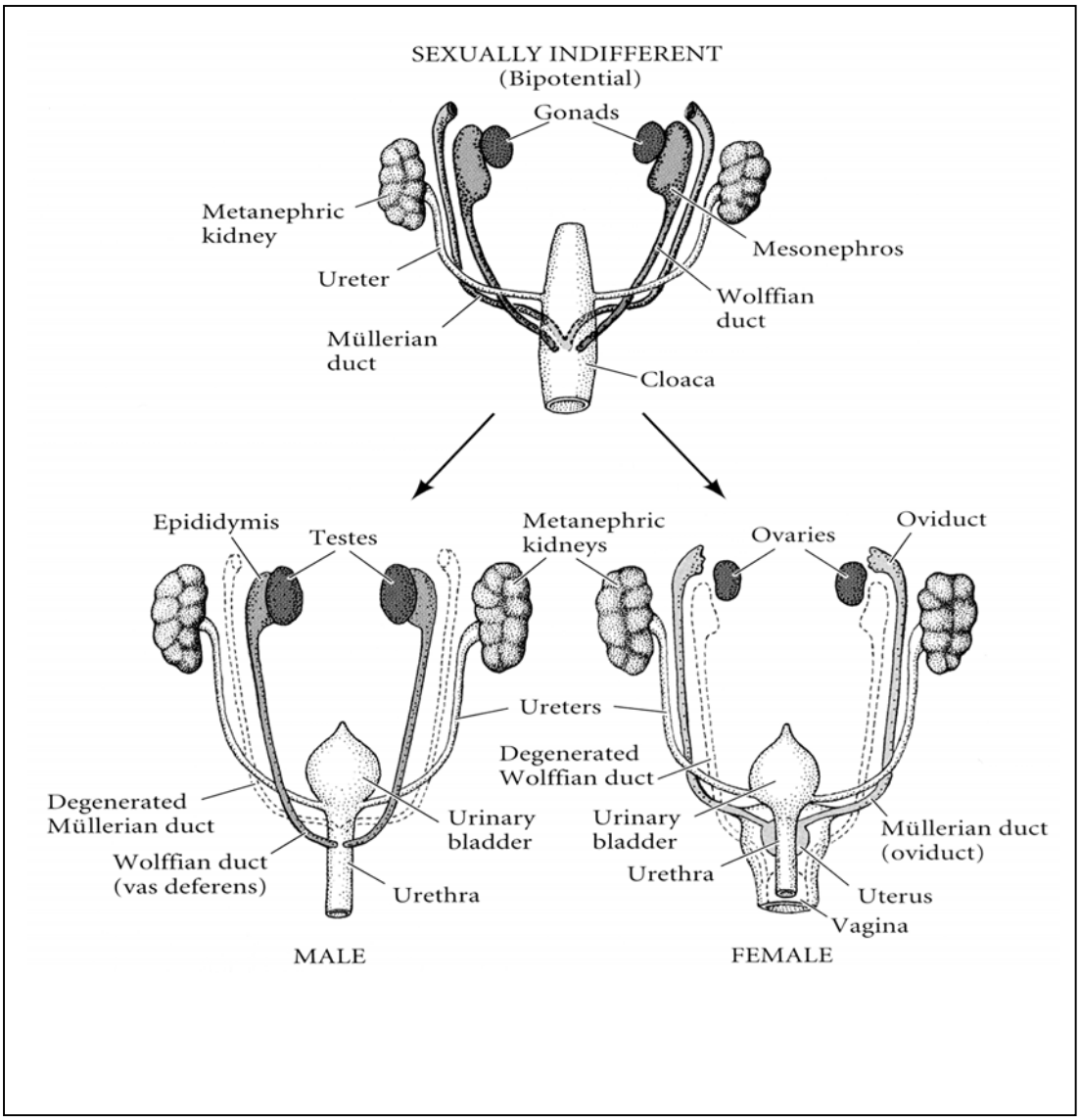


Fig. 2: Sexual differentiation of the bipotential gonad

Interference of endocrine disrupting chemicals (EDC)

If the organism is exposed to endocrine disrupting chemicals (EDC) during this critical period of sex differentiation, this can lead to severe defects like morphological pseudohermaphrodism, which means that the genetic information is reversed in the phenotype, leading to XY-females and XX-males (Schardein 1993). However, EDC do not only generate morphological changes, physiological and behavioral aspects are affected as well (Gray, 1992).

Sexual differentiation of the rat brain

The sexual differentiation of the mammalian brain takes place under the control of gonadal steroid hormones. Action of these hormones during a restricted developmental time period results in permanent changes of hormonal sensitivity of sexually dimorphic regions of the adult brain. Similar to periphery, the developing nervous system of either sex originally is bipotential. Depending on early hormonal influence, the CNS undergoes either feminisation or masculinisation.

Beginning at gestational day (GD) 13 the Leydig cells of the rat testes synthesize androgens; testosterone plasma level reaches a peak at GD 18 (Weirz and Ward, 1980). Via bloodstream testosterone is transported to the brain. Masculinisation processes are initialised in the brain at gestational day 18 and continue during the early postnatal period until approximately the end of the first postnatal week. In nerve cells of special brain regions, such as the medial preoptic area (MPO) and the ventromedial hypothalamus (VMH), the enzyme aromatase transforms testosterone into estradiol, which binds to estrogen receptors (Pilgrim & Hutchison 1994; Lauber & Lichtensteiger, 1994). The peak of estradiol in the brain during this particular time window causes masculinisation and represses feminisation. The activation of estrogen receptors by estradiol regulates gene expression, which in turn influences differentiation processes in neurons, that later result in the establishment of male behavior (masculinisation) and suppression of female behavior and female estrous cycle (defeminisation). The original brain pattern in mammals is female and in order to achieve a male pattern, it has to be masculinised (Gorski, 1971). Male rats castrated between one and five days of age develop behavioral characteristics of genetic females, and if they are injected with estrogen and progesterone as adults, they display female sexual behavior. The influence of steroid hormones on the developing CNS determines not only sexual behavior and gonadotropin release but also many other functions, such as learning behavior and activity patterns, as well as taste preferences and circadian rhythms (Steward, 1975; Valenstein, 1967; Gerall, 1976).

Detailed molecular mechanisms of sexual differentiation in the brain still remain unclear. The study of sexually dimorphic brain regions, as intended in this study, might help to better understand the underlying mechanisms in the development of the brain.

Most features of sexually dimorphic brain functions can be linked to the action of sex hormones during a critical period of brain development. However, it should be noted that there is evidence for an additional direct genetic control of sex differences in certain neuron groups (Sibug et al., 1996, Reisert et al., 1996 and 2002), but also growth and differentiation of such neurons is under control of estrogen (Kuppers et al., 2000). Since (developmental) actions of hormones represent the primary target of endocrine disrupters, we focus on these processes.

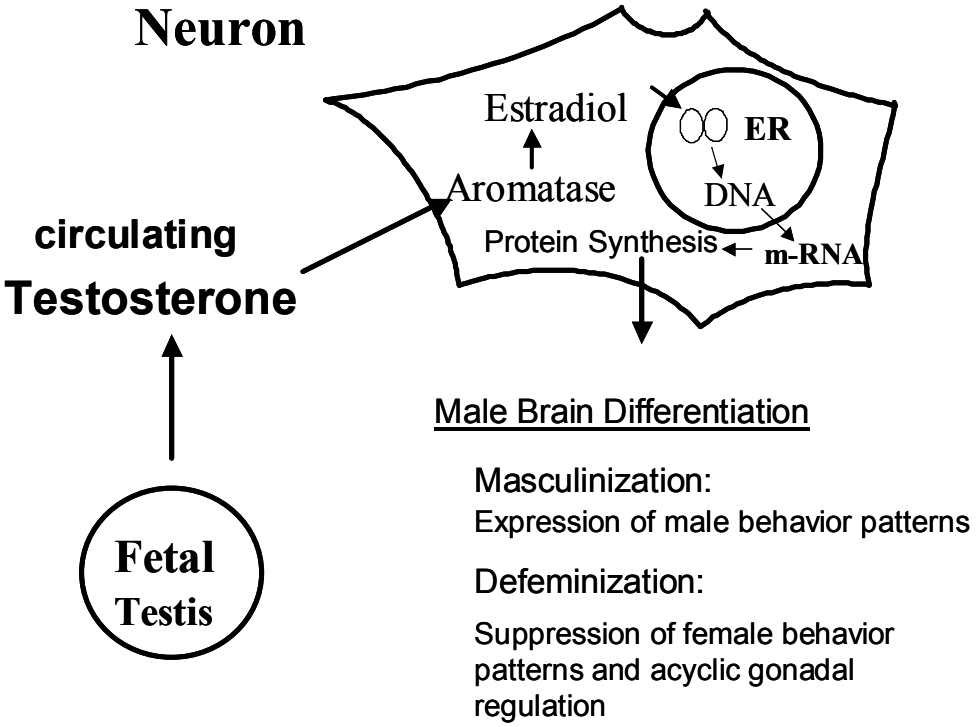


Fig. 3: Action of testosterone on neurons in the central nervous system of rats. Testosterone is synthesized by the fetal testis and is transported via the bloodstream into the brain. In the neurons, the enzyme aromatase converts testosterone into estradiol, which binds to estrogen receptors and acts on the DNA, inducing transcription or repression of target genes. This leads to the sexual brain differentiation, masculinisation and a defeminisation (Fig. taken from Schlumpf & Lichtensteiger, 1996).

Anatomy of the central nervous system of the rat

The characteristic shape of the rat brain (Fig. 4) consists of the two prominent olfactory bulbs, the pear shaped forebrain and the two cerebral hemispheres, which are divided by a deep longitudinal fissure (fissura longitudinalis cerebri). Caudally the cerebrum is separated from the cerebellum by a deep transverse fissure (tentorium cerebelli).

In Fig. 4 the dorsal view (A) shows the olfactory bulbs followed caudally by the olfactory tracts (tractus olfactorius). The ventral view (B) exhibits in the midline the

optic chiasma (chiasma opticum). Caudally to the optic chiasma is, attached to the base of the brain, the pituitary gland (hypophysis). The medulla oblongata is projecting down the spinal cord.

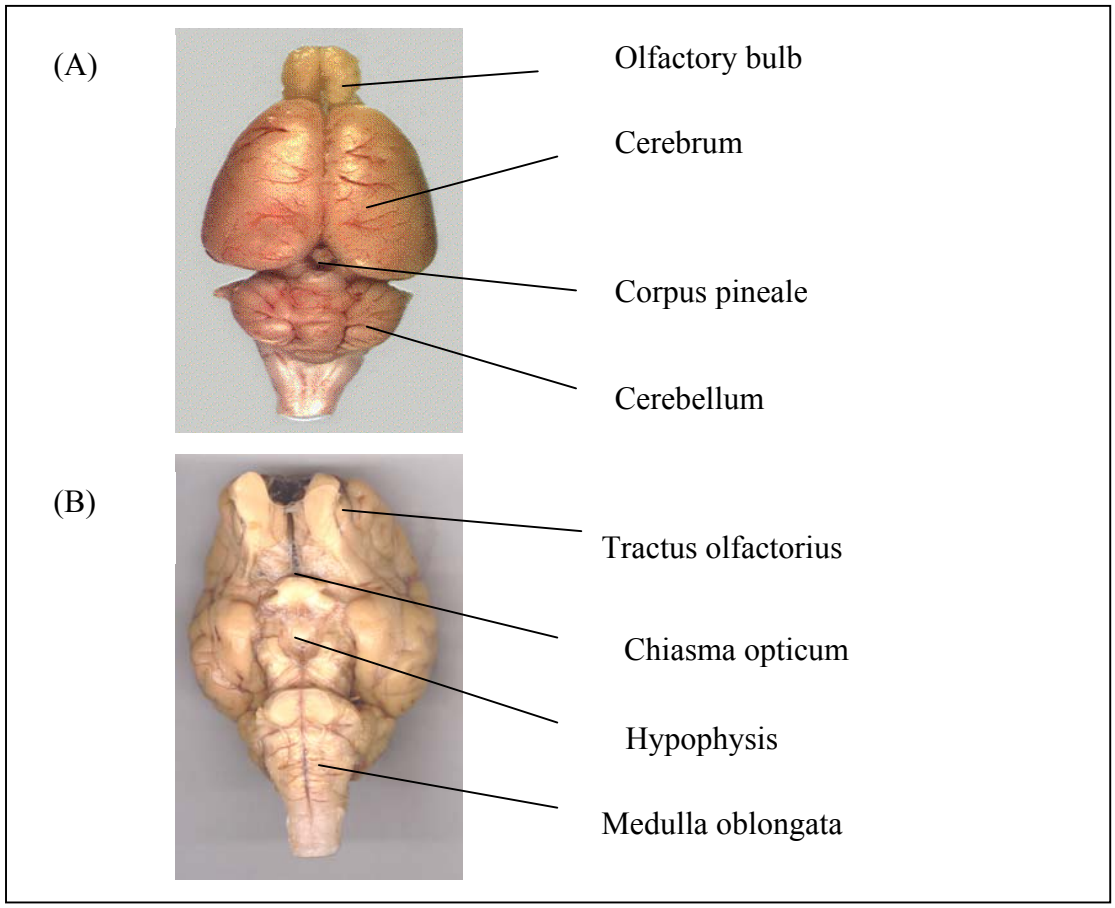


Fig. 4: Dorsal (A) and ventral (B) view of the rat brain (magnification 1.2x)

Sexually dimorphic brain regions

The mammalian brain regionally shows sexual dimorphism. This is reflected by morphological and neurochemical differences between the two sexes, established via exposure to sex steroid hormones during the perinatal period. This study mainly focuses on two brain regions, the ventromedial hypothalamus (VMH) and the medial preoptic area (MPO), which are involved in the control of reproductive behavior and gonadal functions, both in males and females. They are known to be driven by estrogens.

Medial Preoptic area (MPO)

Located at the level of the junction of diencephalon and telencephalon, the medial preoptic area (MPO) is lying ventral to the anterior commissure, close to the hypothalamus (Riley, 1960) (Fig. 5). Anatomically and functionally it connects the

limbic centres with the vegetative hypothalamus. The MPO exhibits a high density of colocalized progesterone-and estrogen receptors (Gorski, 1980). The MPO contains a cell-dense region called the sexually dimorphic nucleus of the medial preoptic area (SDN-POA), which shows sexual dimorphism with regard to the size of certain cell clusters, or the distribution of certain transmitter systems. It is double to five times larger in males than in females. Although its function still remains unclear, it is assumed, that the enhancement of neurogenesis in this region via testosterone action may play a role in masculinisation processes (George 1982). The increased volume of the SDN in males is thought to be induced by estradiol, generated from testosterone, as the expression of the transforming enzyme, aromatase, differs in the SDN of both sexes (Lauber et al., 1997). Research on potential endocrine disruptors has used the volume of SDN-POA as a parameter in female rodents. While the endocrine disruptor bisphenol A had little effect on SDN-POA volume of treated rodents (Nagao et al., 1999), neonatal exposure to diethylstilbestrol (DES) (Faber et al., 1993) and genistein (Faber & Hughes 1991, 1993) increased the SDN-POA volume of genetic females.

The MPO neurons contain more estrogen receptors than any other brain region of both sexes, males and females (Herbison, 1992). The MPO is found to be involved in the regulation of reproductive behaviour (Sodersten, 1985). In males, the MPO is important for copulatory behaviour, whereas in females it is involved in the regulation of maternal behaviour, lordosis and estrous cycle. In males as well as in females electrophysiological activities within the MPO increase during mounting (Kartha, 1996) and lesions at this site enhance lordosis behaviour in females (Powers & Valenstein, 1972) or are hamstring copulatory behavior in males (Klaric, 1985).

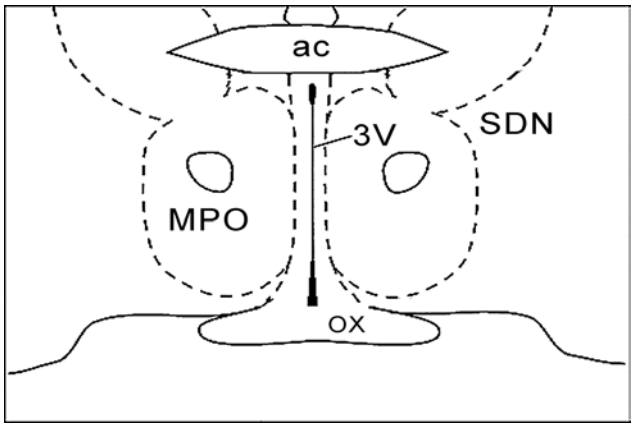


Fig. 5: Schematic figure of the medial preoptic area (MPO), bilateral on the right and left side of the third ventricle (3V). The MPO contains the sexual dimorphic nucleus (SDN).

Ventromedial hypothalamus (VMH)

Ventral to the diencephalon and dorsal to the anterior hypothalamic area, the ventromedial hypothalamic nucleus (VMH) is located (Fig. 6). Like the MPO the VMH is sexually dimorphic in structure and function and it is an important target for regulating action of steroid hormones. Despite the overall volume of the VMH is being larger in males, in females the VMH neurons were found to be larger and to express higher ER alpha levels than in males. The distribution of ER beta, however, does not differ between the sexes, which may also be due to its very low expression level in this region (Ikeda et al, 2003). In behavioural experiments lesions of the VHM disrupted copulatory behavior in female rats (Kennedy, 1964).

The difference in size of the VMH between males and females seems to depend on the estrous cycle; the sexual dimorphism is more clear-cut when female rats are in diestrus (Madeira et al., 2001). The VMH mediates the lordosis behavior in females via modulation of ER and PR (Pfaff, 1980).

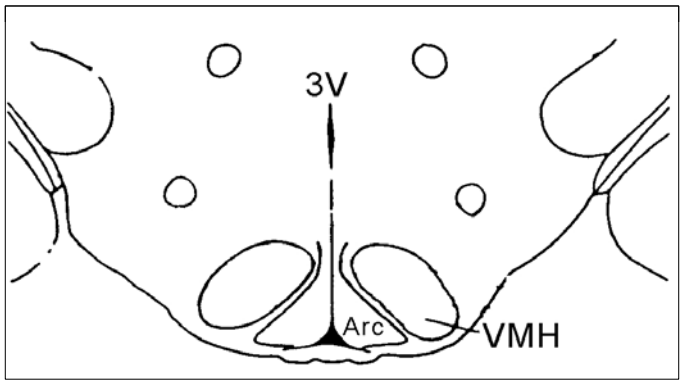


Fig. 6: Schematic figure of the ventromedial hypothalamus (VMH), which is located on both sides of the third ventricle (3V), Arc = Nucleus arcuatus

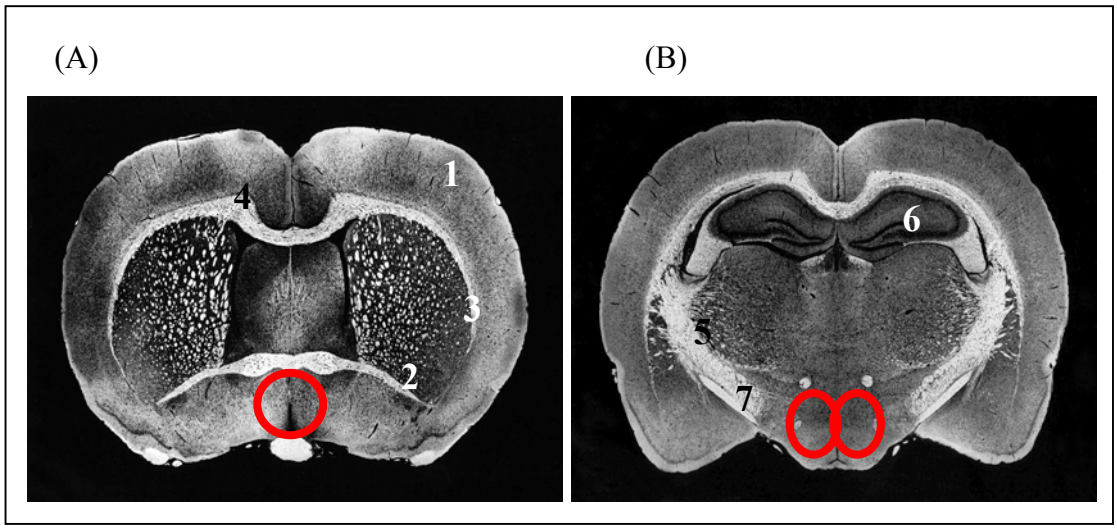


Fig. 7: Frontal brain slides with MPO (A) and VMH (B) (4,84x) 1: Corpus; 2: Commissura anterior; 3: nucleus caudatus putamen; 4: Radiatio corporis callosi; 5: capsula interna; 6: fimbria hippocampi; 7: tractus opticus

Male sexual behavior

Sexual behavior of male rats mainly comprises three behaviors: Mounting (assuming the copulatory position from behind and on the back of the female), intromission (penis is put into the female's vagina) and ejaculation (determined as forceful expulsion of semen). Depending on the female's reaction the male exhibits all three parts of this copulatory behavior or it breaks off before finishing the whole process. Usually, the male only ejaculates, if the female exhibits lordosis behavior. A successful complete mating can be recognized by the fact that the male licks his genitals. Typically rats have multiple ejaculations. In average ejaculation only follows after a number of mounts and intromissions. Multiple measures of the ratio between

mounts, intromissions and ejaculations are used to evaluate sexual motivation and performance.

Female sexual behavior

Female reproductive behavior comprises proceptive (female initiated) as well as receptive (response to the male) behavioral components. In order to display mating behavior a female rat must be sexually receptive (Bradling-Bennet, 1999). The mammalian female formerly was considered as a 'passive recipient' in copulation. Female sexual behavior was measured exclusively in terms of the “lordosis quotient”. Lordosis is a characteristic posture in which the female rodent arches her back to allow the male the copulation act. But recent studies (Erskine, 1989) have shown that this rather passive receptive behavior is only one part of female reproductive behavior. The female rat is also initiating copulation, thus playing a much more active role. In order to attract the male, the female approaches him actively with behaviors such as wiggling ears, hopping and darting in front of her partner. The female even influences the number and timing of copulations by approaching the male and withdrawing from him, provided that enough space is available. This entire and rather complex behavioral pattern is referred to as paced mating behavior.

It has been shown that sexual behavior of the female is regulated by steroid hormones in specific brain regions, such as the VMH (Rajendren, 1991). For example lesions can totally suppress the estrogen-induced lordosis reflex. The most influential regulating hormones for paced mating behavior are estrogens (estradiol) and progesterone. The display of receptive behaviors like lordosis depends on the sequential action of estrogen followed by progesterone. It can be blocked by intracerebral injection of antisense oligonucleotides against Progesterone receptor (PR) mRNA. (Ogawa et al, 1994). Under certain experimental conditions, lordosis reaction can also be facilitated by estrogen alone (Fadem, 1979; Gilman & Hitt 1978). Progesterone also regulates proceptive behavior.

It turns out that the receptive as well as the proceptive behavior components are subject to independent regulation mechanisms in the CNS. This has been proven in studies, where perceptive (lordosis) behavior in ovarectomized hormone-primed females did not show any difference from intact females, whereas proceptive behaviors have been altered in the treated as compared to the control animals (Zipse, 2000).

Females with a mutation of the progesterone receptor are not able to display soliciting behavior and lordosis (Motoyasu 1996). As mentioned above, if antisense oligonucleotides for PR mRNA are injected into the VMH progesterone-facilitated female sexual behavior is being suppressed (Ogawa et al., 1994). The involvement of the MPO in female sexual behavior seems less clear, since application of estrogen as well as lesions of the MPO can increase lordosis responses. Taken together, estrogen shows inhibiting as well as stimulating effects on lordosis and regulates in association with progesterone sexual behavior in females.

Steroid Hormones

Steroid hormones consist of multiple carbon-ring-structures and are generated from cholesterol in the ovary and testis. They are regulated by a feedback-mechanism via the hypothalamus-pituitary-gonad axis (Fig. 10) and regulate a wide variety of cellular mechanisms, like cellular differentiation, cell migration or cell death. For instance in male brain, steroids are responsible for masculinisation processes and initiate neurogenesis and synapse formation.

Estrogens

Estrogens have originally been referred to as the female hormones. They are derived from androgens and in blood are found in relatively higher levels in females as compared to males. There are three different natural estrogens: 17β-estradiol, estriol and estron (Fig. 8). Among them the most potent estrogen is 17β-estradiol (E2), it is produced in the follicle's epithelial cells. In ovaries and, to lower extents in testes, estrogens are produced by de novo synthesis. Additionally estrogens are derived via conversion of androgens by the enzyme aromatase in a wide variety of organs such as brain and fat tissue in both sexes. Estrogens play a major role in the control of both female and male reproduction. They regulate the female menstrual cycle, have an impact on growth and behavior, and affect brain development. Furthermore they regulate morphological, biochemical and electrophysiological matters in numerous brain structures, such as memory, emotions, motor coordination and pain sensitivity (Hampson, 1990, Sherwin, 1998). A small part of estradiol is freely available in the blood, but most of it is bound to a transport protein called the sex-hormone-binding globulin (SHBG).

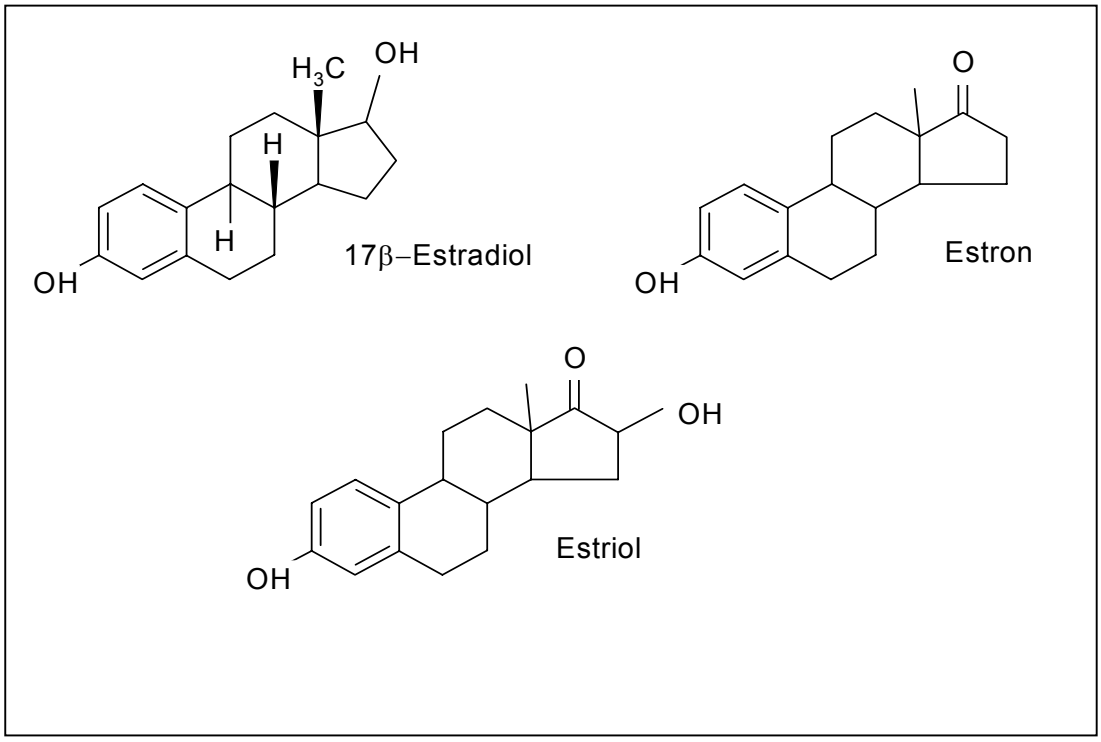


Fig. 8: The three natural estrogens: 17β-estradiol, estron and estriol

Gestagens

The gestagens are a second group of steroid hormones. The gestagen progesterone is involved in the maintenance of pregnancy and the differentiation of the endometrium and the mammary gland and is synthesized in the theca and stroma cells of the ovary during the luteal phase of the estrous cycle. Progesterone is synthesized at several sites such as the adrenal cortex, the corpus luteum, the placenta and the testes. In blood, progesterone is mostly bound to corticosteroid-binding globulin (CBG) or to sex-hormone-binding globulin (SHBG) and to albumin. In rodents the regulating actions of progesterone in the central nervous system (CNS) have been found to be sexually dimorphic (Blaustein et al. 1980; Samama & Aron 1991). It is responsible for proceptive, sexual and maternal behavior. In specific regions of the CNS progesterone receptors (PR) are often associated with estrogen receptors. The PR gene contains a number of estrogen responsive elements (ERE's) in the promoter region (Kraus et al., 1993). Progesterone together with estrogens regulates functions of female reproductive behavior including the induction of lordosis. Furthermore it controls the induction of ovulation. In males, it is found to play a role in the control and mediation of sexual behavior as well (Phelps et al. 1998). Progesterone mRNA expression in brain regions is directly regulated by estradiol

(Shughrue, 1997). It has been hypothesized that a possible mechanism of the brain's estrogen-driven masculinisation during ontogeny is being controlled by progesterone expression in nerve cells (Quadros, 2002).

Androgens

The main site for the synthesis of the most important androgen, testosterone, are the Leydig cells in the testis. Here cholesterol is transformed into testosterone via the intermediates progesterone and androstendione. Androstendione, secreted by the adrenal gland, serves as a further source of androgens. In a variety of male sexual organs like prostate, seminal vesicles and penis, testosterone is transformed by the enzyme 5α-reductase into 5α-dihydrotestosterone (DHT) (Fig. 9). DHT possesses a higher affinity for the androgen receptor. It regulates the differentiation processes in tissues where it is converted from testosterone.

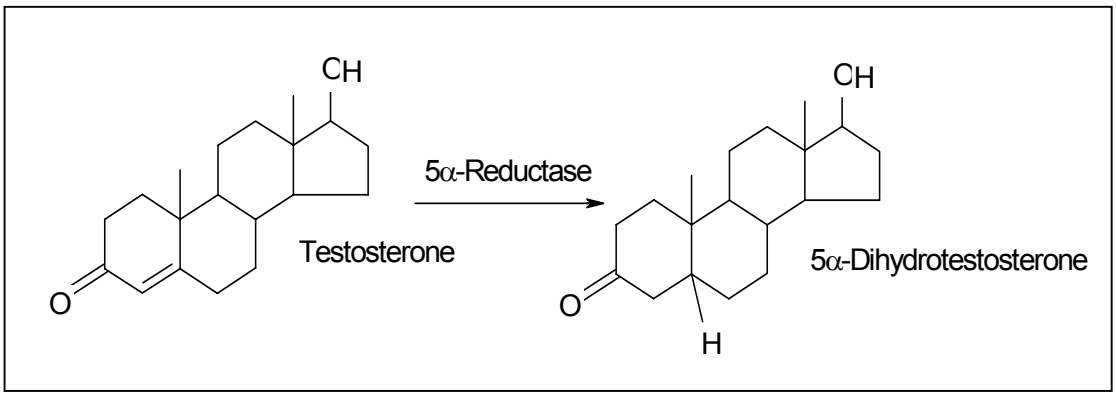


Fig. 9: Transformation of testostreone into 5α-dihydrotestosterone

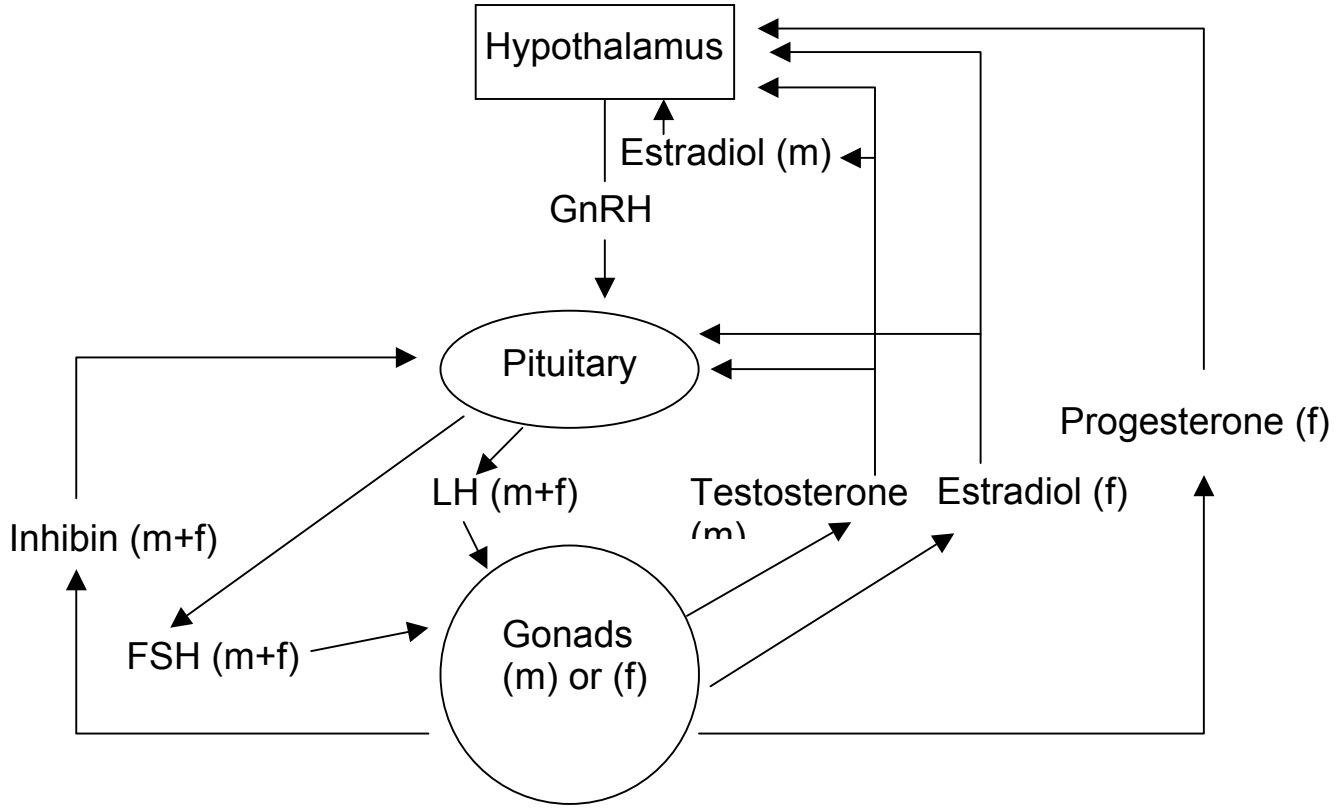


Fig. 10: This figure represents the sex hormone regulation in mammals. Gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the pituitary, which leads to secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two gonadotropins stimulate the gonadal activity. In the male (m) testis, testosterone and inhibin is produced, which regulates secretion of GnRH, LH and FSH from the hypothalamus and pituitary via a feed-back mechanism. In female (f) ovaries secretion of estradiol, progesterone and inhibin is stimulated by FSH and LH. Estrogen stimulates or inhibits hypothalamus and pituitary in females and exerts only a negative feed-back in males. Inhibin inhibits FSH and LH synthesis. Progesterone exerts positive or negative influences on the hypothalamus. At brain level, feedback effects of testosterone are mainly mediated by estradiol locally generated from testosterone, but there also exist androgen-mediated effects.

Estrogen-regulated genes

Steroid hormone receptors

Sex steroid hormones regulate the transcription of selected genes via docking to specific receptors, located either in the cytosol or the cell nucleus. Nuclear steroid hormone receptors all have three main domains: The estrogen receptor (ER) is the main mediator of estrogen- regulated gene expression. It is part of the steroid hormone receptor superfamily of nuclear receptors. The estrogen receptor has a typical structure (Fig. 11). The centre of the amino acid chain contains the DNA

binding domain with a zinc finger- structure. The N-terminal end and the carboxyl-end (CE) of the chain contain transactivation domains (AF1 and AF2) and a ligand-binding domain (LBD), respectively.

N-terminal (AF1)	**DNA-binding domain (DBD)**		**ligand-binding (LBD) and activation domain (AF2)**	**CE**

Fig. 11: Schematic structure of nuclear receptors and their domains

When estrogen binds to the ER, a conformational change causes the dissociation from heat shock proteins (hsp90), and leads to receptor dimerisation. The receptor homodimer then binds to specific hormone receptor palindrome elements (HRE) of the steroid-responsive target gene, estrogen response element (ERE) in the case of estrogen. Nuclear receptor coregulators (coactivators, corepressors) are recruited. Coactivators facilitate local disintegration of nucleosomes by acetylation of histones, and are also involved in transactivation, interacting with the transcription factor complex (TF), which finally leads to activation of RNA polymerase II.

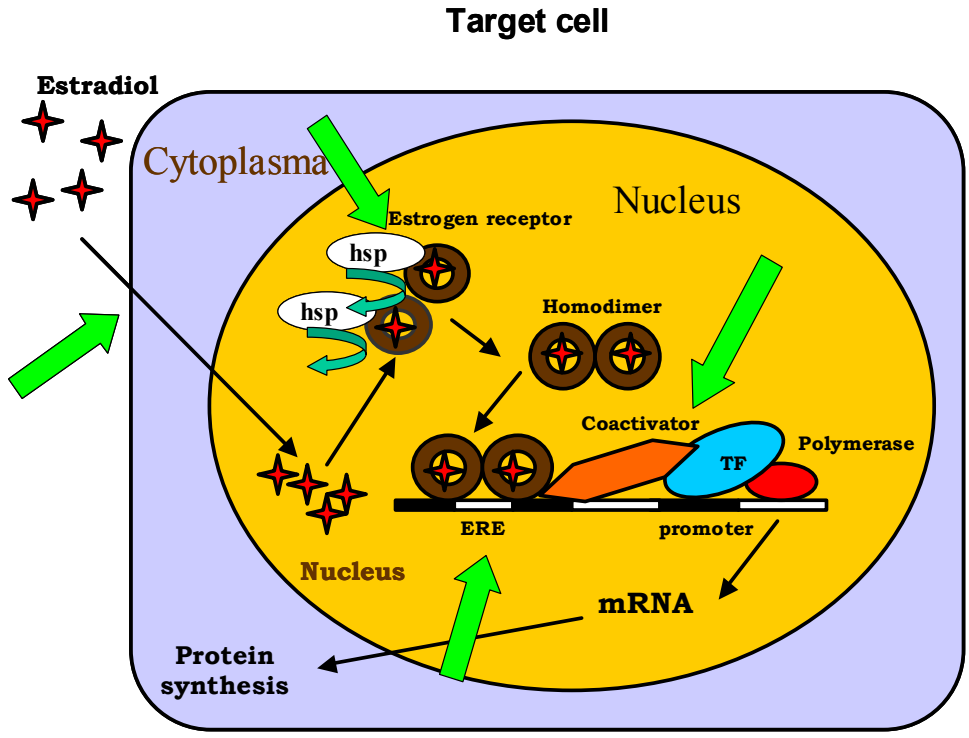


Fig. 12: Molecular mechanisms of steroid hormones (estradiol, red asterisks) or EDC (green arrows)

Estrogen receptors, ERα and ERβ

There are two subtypes of estrogen receptors, ERα and ERβ. Their difference is in the C-terminal ligand-binding domain as well as in the N-terminal activation domain and they are differently distributed among target tissues. ERα has a higher level of expression in uterus, testis, pituitary, ovary, kidney and epididymis, whereas a slightly higher ERβ expression level was found in prostate, ovary, lung, bladder and uterus. Since the distribution of the two estrogen receptors in part is overlapping they may also work together as agonistic components (Barkheim et al, 1998). Yet, the exact roles of the two types of ER claims for further research.

In the brain their distribution pattern is heterogeneous as well. In the ventromedial hypothalamus (VMH) and the arcuate hypothalamic nucleus only ER alpha signals were detected by immunohistochemistry (Ikeda et. al., 2003, Shughrue et al.; 1997, 2001, Pelletier et al., 1988), whereas in the medial preoptic area (MPO), the bed nucleus of the stria terminalis, the cortical nuclei of the amygdala and other regions both, ER alpha and ER beta, have been found. In a number of brain regions ER beta represents the main ER.

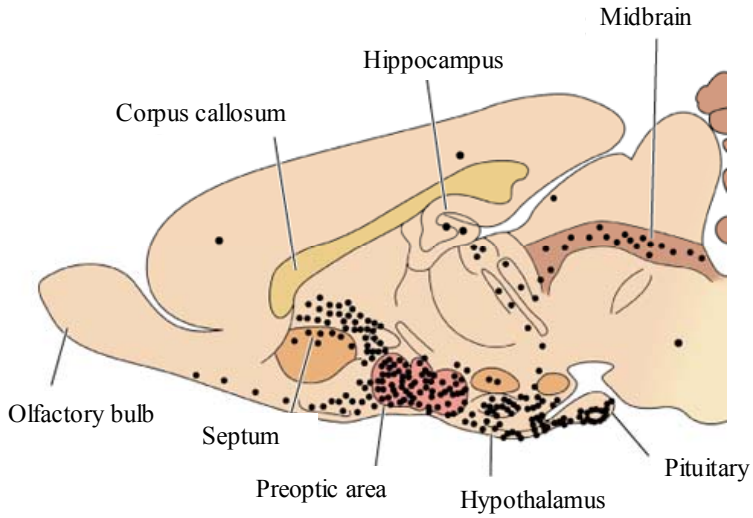


Fig. 13: The different sites of estrogene receptors in the brain

A knockout study has shown that ER alpha in the brain plays some role in reproductive matters (Couse & Korach, 1999). One of ERα's many functions is the control of female sexual behavior and reproduction. ERα knock-out mice revealed a decreased lordosis response and reduced level of parental behavior (Ogawa, 1998). ER beta knockout mice revealed a role of this ER type in brain development (Wang

et al., 2001, 2003). Together with ER alpha, ER beta is involved in sexual brain differentiation and in the control of sexual behavior (Kudwa et al., 2003, 2005).

Progesterone receptor

The progesterone receptor (PR) also is involved in the regulation of reproductive matters. Progesterone receptors play a major role in the induction of lordosis behavior and in the control of luteinising hormone secretion. Induced by estrogens, PR is found in numerous brain regions. Its mRNA expression level is relatively high in the medial preoptic area (MPO) as well as in the ventromedial hypothalamus (VMH) (Hagihara et al., 1992). A remarkable sexual dimorphism was found in the ventrolateral part of the ventromedial hypothalamic nucleus (VMN) in terms of the gene regulation of PR (Brown et al., 1987; Lauber et al., 1991). Progesterone Receptor appears to be significantly more sensitive to estradiol in female VMN as compared to the male one, since low doses of estradiol increased PR mRNA expression in the VMN only in females (Lauber et al., 1991). In the preoptic regions, however PR expression reacted equally to estradiol application in both sexes (Brown et al., 1987).

Preproenkephalin

Preproenkephalin serves as precursor of the neuropeptide met-enkephalin (Noda et al. 1982; Akil et al., 1984). Preproenkephalin mRNA can be found in many regions of the rat brain, comparatively high expression levels are detected in striatum, olfactory tubercle, amygdala and VMH. The PPE gene promoter has two estrogen response elements (ERE) and prolonged application of estrogens raise the PPE mRNA levels in the hypothalamus (Zhu et al., 2001). PPE in the hypothalamus and limbic system is involved in the control of reproductive behaviors such as lordosis (Micevych et al., 1992, Lauber et al., 1990). The regulation of preproenkephalin (PPE) mRNA level in the VMN by estradiol is sexually dimorphic. In ovarectomized rats with average estrogen sensitivity PPE mRNA increased, whereas in orchidectomized males there was no response, neither to estradiol nor to testosterone (Roman et al., 1990).

Endocrine-disrupting chemicals

As stated above, steroid hormone receptors are controlling crucial events in embryonic development and sexual differentiation via their function as ligand-inducible transcription factors. They either activate or repress the transcription of

target genes. The consequences of disrupting these crucial processes can have especially severe consequences if they occur in sensitive periods during development. Additional interactions of xenobiotics are possible at the level of steroid hormone synthesis (e. g., aromatase), metabolism, and transport. The delicately balanced regulatory mechanisms are in danger of being seriously altered, resulting in abnormal differentiation of reproductive organs and central nervous system. In adult age, when hormones have no longer an organizing but an activating or inhibiting function, exposure to EDC may lead to diminished reproductive success and it may harm the fitness of the affected organism. Although first studies on synthetic chemicals and their estrogenic actions have been carried out back in the 1920's (Allen and Doisy, 1923), it is in the 1960´s when the population decline of different bird species in several regions of the United States increased the awareness of these environmentally relevant chemicals. During the past decades there has been an increasingly controversial debate among scientists and the public in terms of possible adverse effects of different chemical compounds found in the environment, suspected to interfere with the regular functioning of the endocrine system. These chemicals therefore have been named "Endocrine-disrupting chemicals (EDC)". At the European workshop on the impact of endocrine disruptors in Weybridge back in 1996 the two following definitions for "endocrine disrupting chemicals" were established:

"An *endocrine disruptor* is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) population."

"A *potential endocrine disruptor* is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) population."

EDC are either of natural origin or generated in industrial production processes. The list of synthetic chemicals is growing exponentially and so is evidence of their increasing occurrence in the environment. The majority of EDC so far identified is lipophilic and have the capacity to bioaccumulate. Because they have hormone-like properties, a large number of these persistent compounds are likely to interfere with physiological pathways in all kinds of organisms.

It therefore is not surprising that several field and laboratory studies have revealed a number of chemicals as contributors to adverse effects in wildlife species and populations. For instance, polychlorinated biphenyls (PCB) have been shown to exert

adverse effects on the reproduction of seals (*Phoca vitulina*) in the Northsea (Germany), leading to a marked decrease of population size (Reijnders 1986).In the middle of the 1980s, anglers in Great Britain reported to have caught fish (*Rutilus rutilus*) with both male and female characteristics. They were found in river waters downstream to sewage outflows. The hermaphroditic characteristics of these fish were hypothesized to originate from the increased release of contraceptives (Purdom et al. 1994). In some regions, the levels of vitellogenin, which normally is only synthesized in the liver of females, reached levels in male fish similar to those of female fish taken from uncontaminated waters. Reijnders and Brasseur reported in 1992 the occurrence of female marine snails (*Nucella lapillus*) carrying male genitalia, including penis and vas deferens. Exposure to the marine antifouling paint tributytltin (TBT) was found to be responsible for these disturbances.

In addition to these findings in wildlife of aquatic ecosystems, harmful effects have also been revealed in terrestrial species. For instance, 90% of the male population of Florida panthers (*Felis concolor*), shows cryptorchidism. And sperm abnormalities in this population exhibit the highest rate ever found in felines (Facemire 1995). Another frequently characteristic example is eggshell thinning and altered gonadal development, the feminisation of male embryos and breeding behaviour abnormalities in birds feeding on insects which were exposed to DDT. (Anderson and Hickey, 1970; Spitzer et al., 1978). Public attention to this entire problem was first raised by Rachel Carson's book “The silent spring”, back in 1962, in which she described fictively the future consequences of bird’s exposure to DDT: extinction of songbirds – a silent spring. It seems conceivable that exposure to environmental endocrine disruptors may account for a large part of widely observed reproductive abnormalities (Facemire 1995).

Besides these reports on the disruption of normal endocrine functions in the fauna of various ecosystems, there is increasing evidence for possible harmful effects on human health. Quite well known end points of suspected effects of EDC are, for example, testicular and prostate cancers in men, reduced male fertility, breast cancer and endometriosis in women, abnormal sexual development, immune suppression, alteration in pituitary and thyroid gland functions and effects on neurobehavioral functions (Crisp et al. 1998).

Nowadays there is a growing number of members of various chemical groups which are suspected EDC, amongst them pesticides, industrial chemicals, and

phytoestrogens. Endocrine disruptors which act in a similar way as the natural estrogens, such as e. g., 17β-estradiol, and therefore are called xenoestrogens (Sonnenschein & Soto 1998), do not necessarily share structural similarities with the natural estrogens. Nevertheless they are capable of evoking agonistic or antagonistic responses in estrogen receptors (Zacharewski 1997).

The first compounds identified as EDC were organochlorines like DDT, PCB's, and Dioxins. Most of them are nowadays prohibited in Europe and North America but many of them are still in use in South America, Africa and Asia. Possibly more important in the future are modern technical products with EDC properties such as surface active chemicals (alkylphenols), plastic monomers (bisphenol A), softeners/plastifiers (phtalates), conservation chemicals, cosmetics, and others. Although the harmful effects on the environment are already proven today, the production of chemicals per year is greater than ever before, reaching actually a level of as much as 400 Mio t. Still very little is known about the effects of single compounds on wildlife, let alone the effects a whole mixture of these chemicals might possibly have.

Possible effects of EDC action on the developing brain

More and more studies focus on EDC and their potential impact on the regulation of target genes via receptor-mediated mechanisms. After binding to the receptor, the EDC may act as full or partial agonists or antagonists. Although they may mimic the action of natural hormones, very often the effect pattern appears to differ from that of natural hormones. The mere complexity of transcriptional regulation by estrogen receptors may explain the absence of a direct correlation between the binding affinity to a receptor and its transactivation. The whole process is influenced by the steroid receptor subtype present, the type of ligand, the available coactivator-corepressor set and finally by the subtype of the hormone response element (Kuiper et al., 1998). Therefore different substances may generate both, agonistic or antagonistic effects. Apart from interactions with hormone receptors, EDC may as well interfere with transport proteins, alter the synthesis and bio-transformation of hormones and interfere with the delicate regulation of the hypothalamus-pituitary gland-secondary glands-axis (see Fig. 12).

Possible consequences of EDC's interactions with developmental processes in the brain differ depending on the sex. A female brain would be expected to develop in a

more masculine way if influenced by an estrogen agonist. The male brain, however, has two possibilities to react. Either the effect of the natural estradiol would be agonistically reinforced by an EDC, which would result in enhanced masculinisation, or a negative feedback effect might occur, that might reduce the testosterone secretion, which would then in turn lead to a reduced level of masculinisation. With regard to the masculinisation process an estrogen receptor antagonist would have the opposite effect. Even more complex situations could occur with partial agonists. There, the result depends on the interactions with the natural sex hormone. If the intracellular concentration of the natural hormone is high, the partial agonist will act like an antagonist, whereas at low concentrations of the endogenous hormone the agonistic activity of the partial agonist would become manifest. Estrogenic organochlorine pesticides have been shown to promote masculinisation processes in male mice (vom Saal et al., 1995). PCB were found to interfere with sexual brain differentiation, affecting the activity of aromatase which converts testosterone into estradiol in neurons (Hany et al., 1999; Crain et al., 1997; Sanderson et al., 2001, 2002). Finally the sensitivity of the brain to estradiol could be changed, e. g. by decreased expression of estrogen receptors.

Sources of Endocrine Disrupting Chemicals

There is a wide range of different sources for chemicals acting as EDC (Tab. 1):

- Medicine
  oncologica (Tamoxifen), contraceptives (Ethinylestradiol)
- Agriculture
  herbicides, fungicides, insecticides (o, p'-DDT)
- Industry
  flame retardants (PBDE 99), plastic components (Bisphenol A)
- Plants
  phytoestrogens (Genistein, Coumesterol)
- Cosmetics
  UV Filter (4-MBC, 3-BC)

Tab. 1: Possible sources of EDC

Based on their similar molecular structure to 17β- Estradiol, the compounds were divided into 6 groups: Diethylstilbestrol (DES)-like chemicals, steroids, phytoestrogens, diphenylmethans, biphenyls and phenols. The binding activity was defined relatively to that of 17β- Estradiol. (Fang et al, 2001)

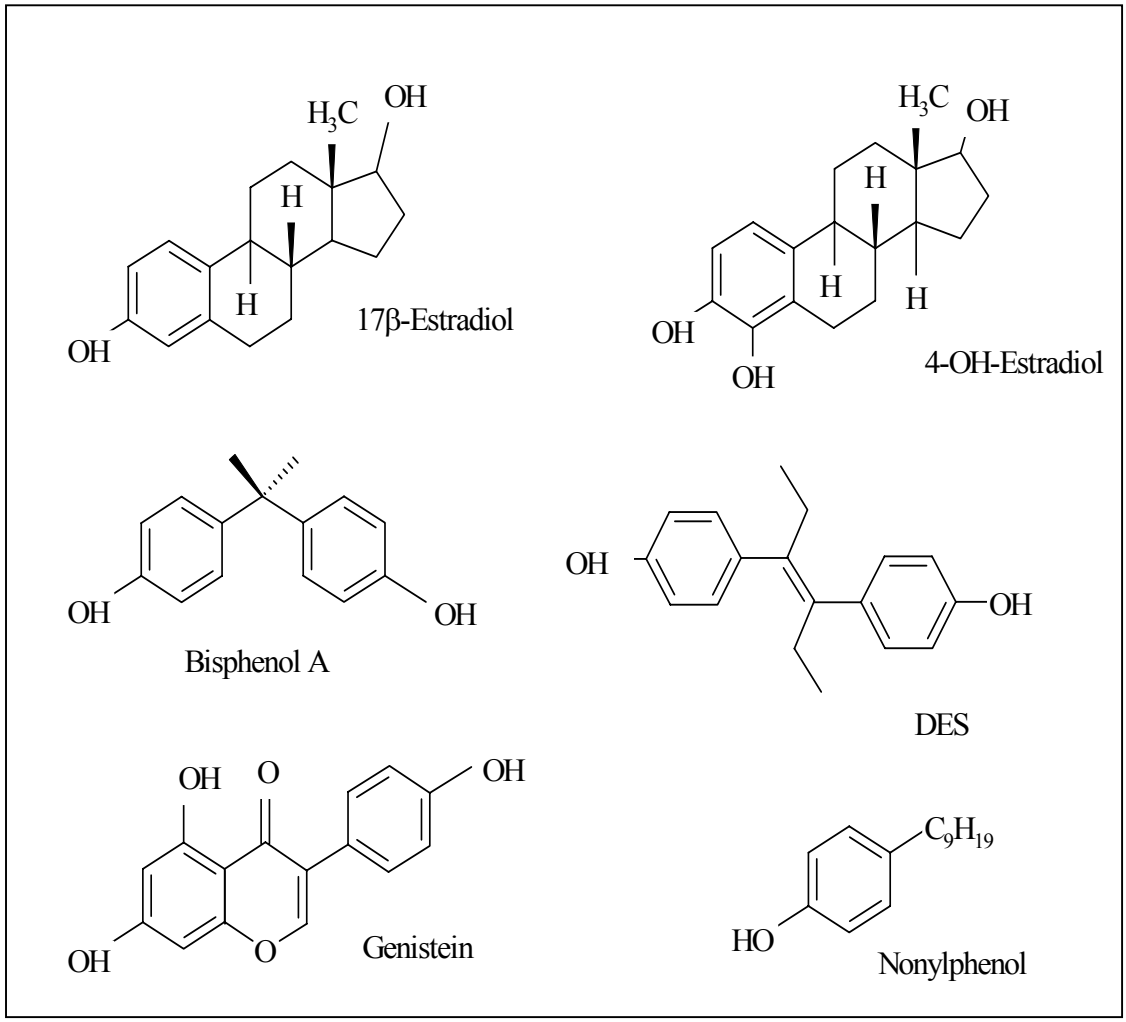


Fig. 14: Molecular structures of endocrine disrupting chemicals compared to 17β-Estradiol

Another possible source of EDC among the huge number of halogenated organic chemicals are the increasingly important polybrominated flame retardants. Structurally related to the well known PCB they are suspected to have endocrine disrupting potential as well. They are in the focus of the present study. The last chapter of the introduction thus deals with this lately arising group of chemicals

Flame Retardants

Since mankind discovered the benefits of fire there has as well always been the danger of sever burning damages on human property. Therefore big effort has been done on finding methods to lower the risk of inflammation of materials. With the invention of chemical flame retardants a great breakthrough has been achieved, however as e. g. this study shows, the compounds in use do not only have beneficial properties. Especially in terms of risk for the environment the long durability of flame retardants causes major problems, as they are only very little biodegradable. These flame retardants are used as additives in all kinds of polymer materials or are simply applied to a material in order to increase the fire resistance of the particular product. Within the past 50 years the use of polymers with different properties and applications has increased exponentially and they are now found almost everywhere, e. g. in clothing, toys, furniture, cars, computers, etc. As these polymers are petroleum-based and hence are flammable, flame retardants are applied to combustible materials such as plastics, wood, paper, and textiles, in order to heighten their flashing point.

Brominated Flame Retardants

Due to its heat stability the halogen brome is thought to function as a fire-retardant compound. Brominated flame retardants (BFR) are produced via direct brominating of organic molecules or via addition of bromine to alkenes and are used in large quantities in a wide variety of consumer products.
Brominated flame retardants can be subdivided into three groups, depending on the way how they are incorporated into the polymer structure: brominated monomers, reactive flame retardants and additive flame retardants.

Brominated polymers are produced via polymerisation of prior brominated monomers like styrene or butadiene. The subsequent polymerisation process results in a polymer containing both brominated and non-brominated monomers. Reactive flame retardants, such as tetrabromobisphenol A, are chemically bonded into the plastics, whereas additive flame retardants, including polybrominated diphenyl ethers (PBDE), are simply mixed with the other polymers, and therefore are more likely to evaporate from the products (Hutzinger et al., 1987).

Polybrominated diphenyl ethers

According to the latest information available (Darnerud et al., 2001) the actual global production is around 40,000 tons a year. PBDE are subdivided into three groups according to the degree of bromination, i.e. Penta-BDE, Octa-BDE and Deca-BDE and classified after their average bromine content. They are relatively resistant towards physical, chemical, and biological degradation. Their boiling point lies between 310°C and 425°C and at room temperature they have a low vapour pressure. The higher brominated PBDE are, the more lipophilic and the less water soluble they become.

PBDE are mainly used in textile material and in plastic coatings, with concentrations ranging from 5% up to as much as 30%. The general chemical formula is $C12H_xBr_yO$ with hydrogen and bromine atoms always being 10 in summary. Within the carbohydrate ground structure any of the 10 hydrogen atoms can be exchanged with bromine. Thus 209 congeners are possible and according to the nomenclature of the structurally related PCB divided into 10 congener groups.

Penta-brominated diphenyl ethers

PBDE99, the compound of interest in this study, is one of the two most commonly used congeners. There are mainly three low brominated PBDE: 2,2',4,4'-tetrabromodiphenyl-ether (BDE-47) and 2,2',4,4',5-pentabromodiphenyl-ether (BDE-99) and 2,2',4,4',6-penta-BDE (BDE-100) in commercial products. BDE-99 and to a lower extent BDE-100 are produced via continuous bromination of the ground product BDE-47. A commercial mixture often consists of 35% BDE-99 and 6.8% BDE-100, the rest made up by BDE-47. The ortho-para directing property of the bromine atoms in BDE-47 and steric hindrance might be the reason for the higher amount of BDE-99. All three are of particular interest, because they are found in a rising number of biological samples including human tissue (de Witt, 2002).

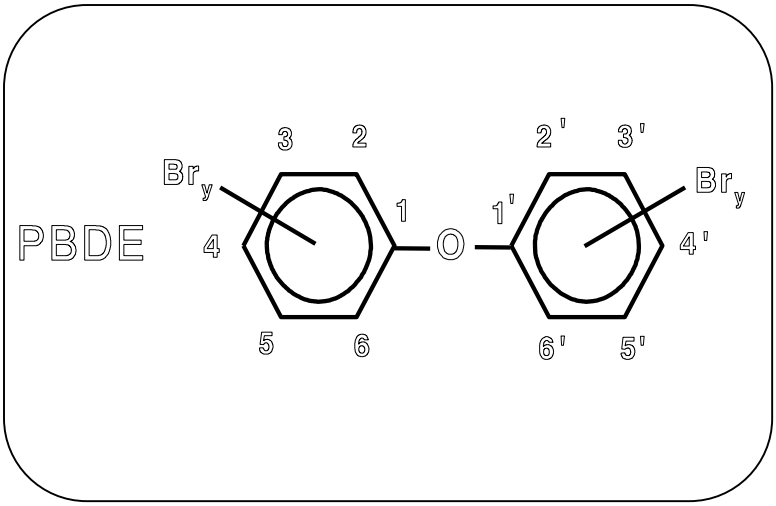
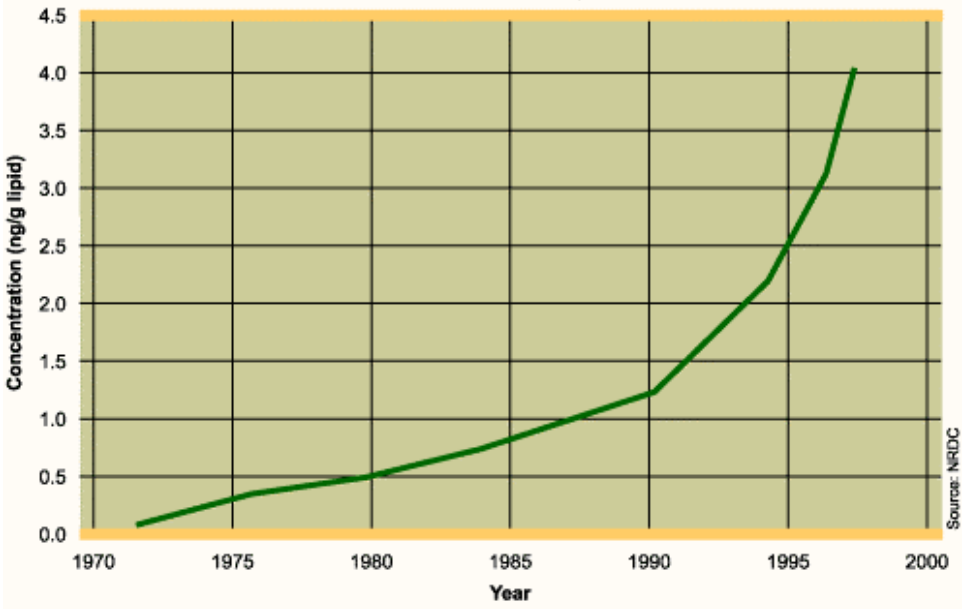


Fig. 15: The general formula of polybrominated diphenylethers

The polychlorinated biphenyls (PCB) are well known to bio-concentrate and bio-accumulate in the environment. Because of their structural similarity, their high affinity to particles and their lipophilic characteristics, PBDE have been suspected to behave and act in a manner similar to that of PCB (Pijnenburg et al., 1995). In fact PBDE have been found in various biotic samples from birds, seals, whales as well as in human samples such as blood, adipose tissue and breast milk (de Boer et al., 1998; Stanley et al., 1991)

How important PBDE have become as environmental contaminants has been shown in a study from Sweden, where the accumulation in human breast milk, with constantly increasing concentrations within the last 30 years has been revealed. Starting in 1972 at a level of as low as 0.07 ng/g lipid weight rising to as high as 4.02 ng/g lipid weight in 1998 (Meyronite et al., 1999). Even higher levels have been detected in human samples from the USA. There human adipose tissue and milk levels of PBDE99 range between 0.01 and 0.3 µg/g lipid with upper levels up to 0.11 µg/g lipid (She et al., 2002; Schecter et al., 2003; Sjödin et al., 2003) while European levels are considerably lower (Sjödin et al., 2003). For Californian harbour seal blubber, a mean PBDE99 concentration of 0.112 µg/g lipid was reported, with a range up to 0.303 µg/g lipid (She et al., 2002).

Figure 34
PBDEs in Breast Milk, Sweden



It is true that PCB concentrations in wildlife are still higher than those of PBDE, but after being prohibited some 20 years ago the former are declining over the same time period, while levels of the latter compounds are rising.

Until today, thyroid function has been considered as one of the most sensitive end points of PBDE toxicity in vivo. Induction of thyroid hyperplasia and alteration of thyroid hormone production have been observed in rats and mice (Darnerud et al., 1996; Fowels et al., 1996; Meerts et al., 2000) The fact that these phenomena have also been observed for other organohalogen compounds with similar structure: the PCB and their hydroxylated metabolites (Brucker-Davis, 1998) is a further matter of concern and should generate additional research on that issue. The dioxin-like, Ah-receptor-mediated induction of cytochrome P450 1A1 and 1A2 in vitro (Brouwer et al., 1998) and in vivo (Hamberg et al., 1998) is another characteristic that PBDE have in common with PCB and the polybrominated biphenyls (PBB). Last but not least the metabolised forms of PBDE (after hydroxylation in the liver) possess potential biological importance (Meerts et al., 2000).

Aim of the present study

Brominated flame retardants have been identified as environmental contaminants with increasing importance on a global scale. Bioaccumulation was demonstrated in marine mammals, birds and humans for polybrominated diphenyl ethers (PBDE). During the last 10 years, levels in human breast milk have risen to about 4 ng/g lipid. Potential targets of environmental chemicals, that are of major importance to populations are reproduction and ontogeny. Information on the toxicity of PBDE is scarce. However, preliminary experimental evidence suggests adverse effects on the developing nervous system. The present investigation is part of a European Union (EU) project designed to clarify the potential developmental neurotoxicity of PBDE. The pentabromo-diphenyl ether, PBDE99, was chosen, because it is one of the congeners typically present in the biosphere. Since PBDE were suspected to have effects similar to PCB, which have been shown to interfere with brain development, a commercial PCB mixture (Aroclor 1254) was chosen for a comparative study. Certain PCB congeners are known to be active at estrogen receptors, and PCB mixtures have been found to interfere with reproductive and sexual differentiation processes (see Hany et al., 1999). In vitro data suggest that certain PBDE may also interact with estrogen receptors (Meerts et al., 2001). Therefore, PBDE99 was studied for possible effects on neuroendocrine development. The embryo and fetus is particularly at risk with respect to adverse effects of xenoestrogens, because it develops in an environment with low endogenous estrogen levels.

While most of the maternal estradiol does not normally interfere with sexual differentiation of the brain, because it is bound to transport proteins and does not have access to the fetal circulation, xenoestrogens are bound less tightly to specific binding proteins and due to their lipophilicity, readily cross the placenta barrier, enter the embryonic circulation and diffuse into the embryonic brain, which lacks a blood brain barrier. This way they have the opportunity to interact with receptors in an environment with low levels of endogenous estrogen. This may result in altered sexual development.

In the present study, the developmental long-term effect of the polybrominated flame retardant PBDE99 was analyzed in two sexually dimorphic brain regions of adult rat offspring, the ventromedial hypothalamus (VMH) and the medial preoptic area (MPO).

We hypothesized that expression and estrogen sensitivity of estrogen target genes may be permanently changed by exposure to endocrine active chemicals during ontogeny, leading to alterations in sexually dimorphic brain functions. We studied the expression of estrogen receptor alpha and beta, progesterone receptor (PR) and preproenkephalin (PPE), the precursor of enkephalin. PR and PPE were chosen as parameters of estrogen sensitivity.

Time-pregnant Long Evans rats were treated subcutaneously with either PBDE99 (2 dose level) or Aroclor 1254 during a defined part of gestation. The subcutaneous route was chosen, because the amount of highly pure PBDE99 was limited. During postnatal ontogeny, general developmental parameters as well as parameters linked with sexual development (i.e. onset of puberty) were monitored in the offspring. In adult offspring, expression of mRNAs encoding for PR, PPE and estrogen receptors α and β1 was quantified in medial preoptic area and ventromedial hypothalamic nucleus by Real Time-PCR. In order to assess possible changes in the sensitivity of target genes, part of the male and female offspring was gonadectomized in order to reduce and stabilize endogenous hormone levels, and injected with one dose of estradiol. The study is expected to provide information on effects of PBDE on sexual brain differentiation and, on a more general basis, on the interaction of xenobiotics with sexual differentiation processes.

This represents a novel approach to identify possible developmental effects of xenoestrogens in the central nervous system at a molecular level. Behavioral studies can then link the molecular findings to the organismic level. Exposure to chemicals during sexual differentiation is a matter of concern, because if it takes place during sensitive phases, the development of the central nervous system, as well as of the reproductive system, could be harmfully affected by exogenous substances even below levels of general toxicity. And, while the chemical exposure may be transient, its effects may result in permanent alterations of brain structure and function, if the exposition takes place within sensitive periods in development.

Some ethical considerations

The use of animals for this study requires some observations on the justification for this purpose. The aim is to balance the misfits done to the creature with the benefit expected from this study. Only after due deliberation it is appropriate to interfere with the right of any creature for integrity and life. For the sake of simplicity the following considerations are not meant to discuss ethical matters in detail, as there exist

already many articles on that issue. In my opinion ethical decisions are always a matter of personal perspectives, so this chapter only gives a personal statement on how the ethical dilemma discussed above has been handled in this study.
For achieving the aims of this study, animal experimentations had to be carried out. What are animal experiments? A frequently used definition is, these are "experiments, that do not serve the individual animal which is taken for to carry out the experiment". That means the particular animal does not have any benefit from the experiment it is used for. This certainly is the case in the present study. Why was it necessary to use animals? The results of this study demonstrate that not all aspects of toxicology can be assessed in in-vitro systems. The effects of PBDE99 could not have been predicted from in vitro data. An animal's body is a three-dimensional system with interacting physiological systems, whereas in-vitro systems only are able to give first insights into certain chemicals' impact on selected mechanistic questions. The metabolism of an animal may change the chemical´s property, e.g. by making it biologically active through liver metabolism.
Toxicological matters are a special issue with regard to the use of animals. One can only choose between two evils: not to gain any knowledge about impact of chemicals on nature and mankind by deciding to go without animal experiments. Or, as this is fairly unacceptable, to use animal models to learn at least something about effects of chemicals on living organisms, in order to assess the risk posed by the use of chemicals.
Once the decision has been taken to use animals for a study, the scientist's responsibility comprises all aspects of the well being of the handled creatures. In order to standardize this, "International Guiding Principles for Biomedical Research Involving Animals" were formulated in 1985 by The Council for International Organizations of Medical Sciences – CIOMS (News Physiol. Sci. 17, Febr. 02, 43-46). The present investigation was conducted according to the Swiss Law for the Protection of Animals and the Guidelines of the Swiss Academy for Medical Sciences.
In principle the guidelines state that the extent of animal suffering is based on human analogy. The assessment of the degree of pain is done by watching the animal before, during and after the experiment. Pain should be reduced as much as possible (in terms of the rules given by the 3R = reduce, replace, refine) to achieve the results. In this study, the doses of chemicals applied did not cause clinical signs of toxicity.

The animals, that were studied for female sexual behavior were not gonadectomized. Formerly ovariectomy followed by treatment with estrogen and progesterone was a standard method to create a standardized condition independent of the physiological estrous cycle. Instead of this invasive method in this study the less stressing and not at all painful method of taking vaginal smears was chosen to ensure that females were taken for the behavioral studies always at the same stage of the estrous cycle (and thus similar hormone milieu).

If operative methods have been found to be necessary (e.g. for the estrogen challenge part) the surgical manipulation was carried out under anesthesia followed by analgesic medication.

To meet further the needs of the rats used in this study, they were housed in groups, as rats are social animals, who like to interact with each others. A standard number of 2-3 adult males or, due to their lower size, 3-4 females were housed in one cage. Furthermore, in terms of “behavioral enrichment”, they were given the opportunity to carry out some of their natural behaviors (exploring the environment, seeking for shelter, etc.) by adding plastic rings into the cage. These rings were very well accepted by the animals as they were intensely used to climb on them, to rally around them, or to sleep in groups inside of them.

In order to reduce individual stress, the animals were given a period of adjustment to handling before using them for experiments.

Finally the question, whether or not it is justified at all to use animals for experiments in environmental toxicology, is in some way obsolete, since in fact all wild living animals involuntarily undergo a huge experiment on the impact of chemicals on their development as indicated by the high levels of chemicals found in a variety of samples from wild animals. The fact that the right of any creature for integrity and life is endangered by the massive release of chemicals into the environment by humanity, seems to make it indispensable to use animals to learn more about the risk of chemicals. Such data from animal experiments serve also the interest of animals.

MATERIALS AND METHODS

A. Animals

Fig 16 Long Evans rat

Long Evans rats (Fig. 16) were bred in our own colony at the Institute of Pharmacology and Toxicology, University of Zurich, maintained by the Institute of Laboratory Animal Science. The stem of Long Evans rats has been developed in 1915 by Long and Evans. They bred Wistar albino females with males of the wildtype *Rattus norvegicus* leading to the smart rats with mainly white fur but a dark head and a spotted dark line on the back.

The animals were kept in groups in Macrolon cages (cage type 4, 19 x 38 x 60 cm), with a maximum number of 4 females or 3 males. The animal room had a controlled light cycle of 14 hours light (2:00 am – 4:00 pm), constant temperature (22 - 23° C), and around 40 - 58% relative humidity. Food pellets (Provimi Kliba, Kaiseraugst, Switzerland, chow no. 3340) and tapwater was given ad libitum. Rats were marked with spots of picric acid.

Mating/Birth

One receptive untreated female (3-6 months old) was mated with an untreated male over night, starting at 4:00 pm. Early next morning, the male was removed and, a vaginal smear was taken from the female, to check for presence of sperms. Females with sperm-positive smears were kept in groups of up to 4 animals, but separated one day before the expected parturition. The day of positive vaginal smear was defined as gestational day 1 (GD 1). Pups were born at GD 23, which was defined as postnatal day 1 (PN 1). In order to standardize postnatal conditions (feeding, etc.), littersize was adjusted to 8 to 10 pups. If necessary, surplus pups were marked and

added to litters with less than 8 pups. These additional pups were not used for further analyses. Weaning and separation of sexes was carried out on PN 28.

B. Chemicals

PBDE 99 (2,2',4,4',5-Pentabromodiphenylether, purity > 99%) and Aroclor 1254 (commercial PCB-mixture) were obtained from Promochem GmbH, Wesel, Germany.

C. Treatment

PBDE99 was dissolved in a few drops of toluene and further dissolved in olive oil, Aroclor 1254 was dissolved in olive oil. The toluene from the PBDE99 solution was evaporated over night on a magnetic stirrer. Olive oil served as vehicle control.

Time-pregnant rats were subcutaneously injected daily with PBDE 99 (1 or 10mg/kg/day), Aroclor 1254 (10 or 30 mg/kg/day), or vehicle (olive oil) from gestational day (GD) 10 to GD 18 (9 injections). Injections were carried out in the late morning.

C 1. Steady state conditions (Baseline)

In the baseline experiment under steady state conditions, the F1 offspring received no postnatal treatment. Male and female offspring were sacrificed at 4 months of age (120 days). The estrous cycle of females was monitored in the morning by daily vaginal smears, females were always sacrificed in metestrus (= diestrus 1). It should be noted that the offspring continued to be exposed to either PBDE99 or Aroclor 1254, since both bioaccumulate. Exposure to PBDE99 was confirmed at 120 days of age by chemical-analytical analysis.

Schematic overview: Baseline experiment

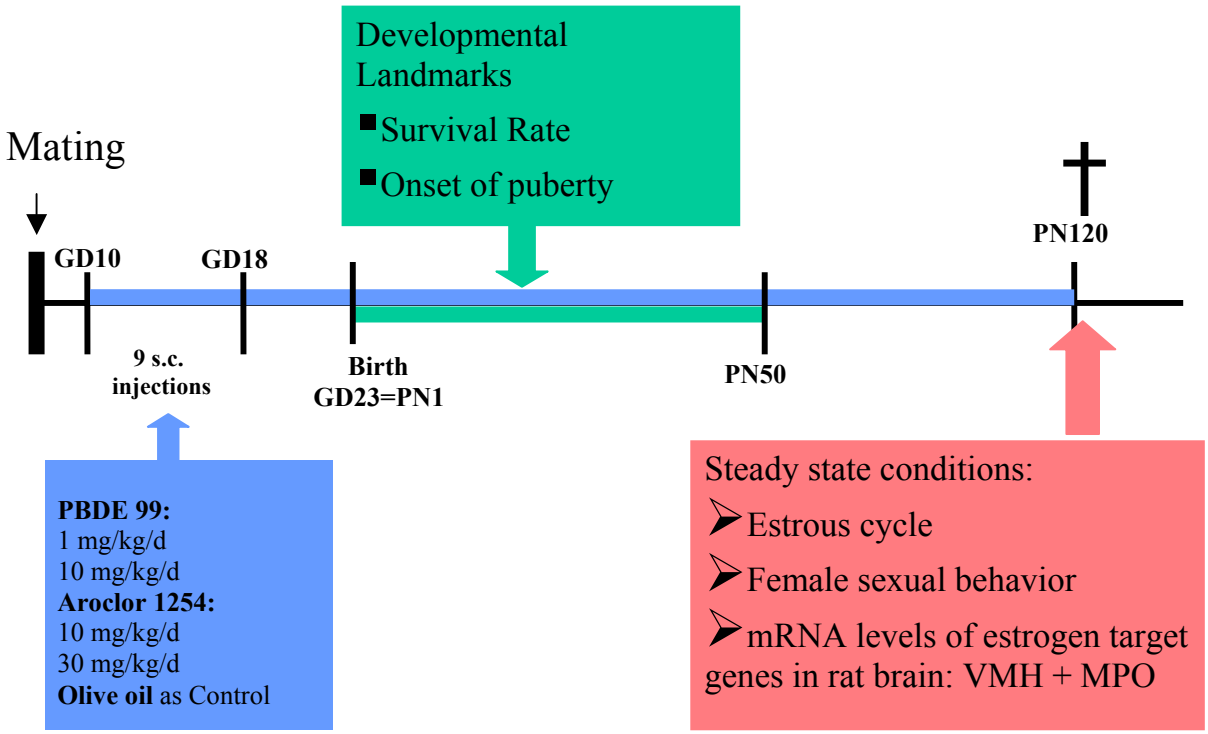


Fig. 20 Study design for steady state conditions. The parental generation was exposed to the test compounds by 9 s.c. injections from gestational day 10 to 18.

C 2. Acute estrogen challenge

At PN 70 = 10 weeks of age the F1-animals were gonadectomized. After a recovery period of 2 weeks, the animals were given a single subcutaneous injection of either 17β-Estradiol (E2) (Calbiochem Lucerne, Switzerland, dissolved in a few drops of ethanol and oliveoil) 10 µg/kg bodyweight, or of the vehicle. 6 hours later the animals were sacrificed.

Schematic overview: Acute estrogen challenge experiment

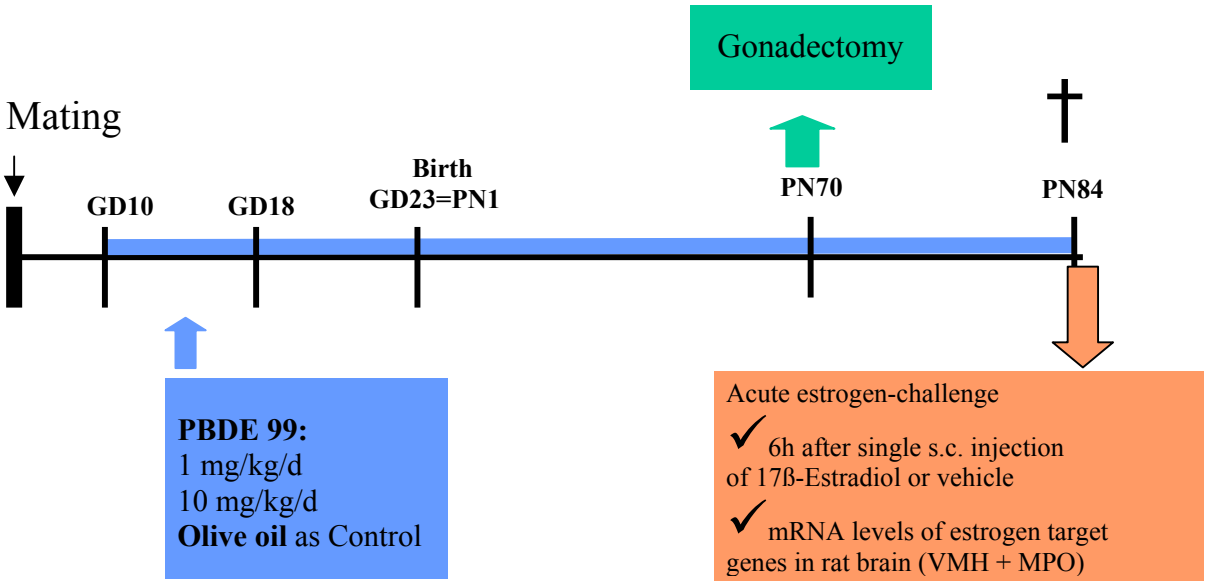


Fig. 21 Study design of the acute estrogen challenge. The animals were exposed to the test compounds in the same way as the steady state animals. On PN 70 (at the age of 10 weeks) F1-animals were gonadectomized. The animals were allowed a recovery time of two weeks and then injected with vehicle or estradiol (10 µg/kg s.c.) and killed six hours after injection.

For gonadectomy the offspring were anesthetized with a combination of fentanyl, fluanisol, meditomidine and atropine according to the protocol of the Institute of Laboratory Animal Sciences, University Zurich (Table 2). Injection volume was 150µl/100g body weight s.c..

Component	**Volume 10 ml**
Fentanyl citrate and fluanisone (Hypnorm, Janssen)	1,25 ml
Medetomidine (Domitor, Pfizer)	1,25 ml
Atropinsulfate 0,1% (Atropin, Kantonsapotheke Zurich)	0,25 ml
Tap water	7,25 ml

Tab. 2 components of anesthetic cocktail

Females were placed on the side on a sterilized plate. The skin was disinfected with 70% ethanol. On either side a 10 mm craniocaudal skin cut was made and the abdominal muscles were divided. The ovaries were taken out and pinched off from the fallopian tube with a clamp before truncating. Subsequently the muscles and the skin were sown up (Supramid 1.5 metric DS19, B. Braun Medical AG, Switzerland). Males were placed on their back on the sterilized plate. The skin of the scrotum was disinfected, in the middle of the scrotum a 10 mm cut was made with a scalpel and

for both sides the pellicle around the testis was removed. Than the testes were pulled out and pinched off with a clamp. On each side a ligation of the vas deferens was made, in order to prevent blood loss, before cutting off the testis. Subsequently the ligated end was placed back into the scrotum, which was then closed with two nodes of thread.

D. Developmental analysis

After birth different developmental endpoints were investigated at fixed ages such as weight gain, righting behaviour, anogenital distance.

Protocol:

- At postnatal day 2 (PN 2), 4, 6, 8, 9, 12, 13, 14 and 15 the pups were weighed.
- At PN 2, 4, 6, 8 and 9 righting behaviour was tested.
- At PN 2, 6 and 14 anogenital distance was measured.
- The opening of eyes was checked from PN 12 until the complete opening of both eyes.
- At PN 28 the offspring were weaned from the mother. Males and females were housed separately with a maximum number of 3 to 4 per sex (minimum 2, because rats are social animals).
- The onset of puberty was studied in females from PN 34 (vaginal opening) and from PN 41 in males (preputial separation).

Estrous Cycle

The estrous cycle of rats normally comprises four days and is characterized as proestrus (Pro), estrus (Est), metestrus (diestrus 1 = D1) and diestrus (diestrus 2 = D2) identified by cytology of vaginal smears (Long and Evans, 1922, Marcondes et al. 2002) (Figs. 17 and 18). Vaginal smears were taken every day at around noon, stained with toluidinblue, and examined to determine the stage of the estrous cycle (Fig. 18). Ovulation occurs during the night between proestrus and estrus. During this time, the female is receptive and will accept males. To standardize experimental conditions, only females in proestrus were taken for behavioural experiments.

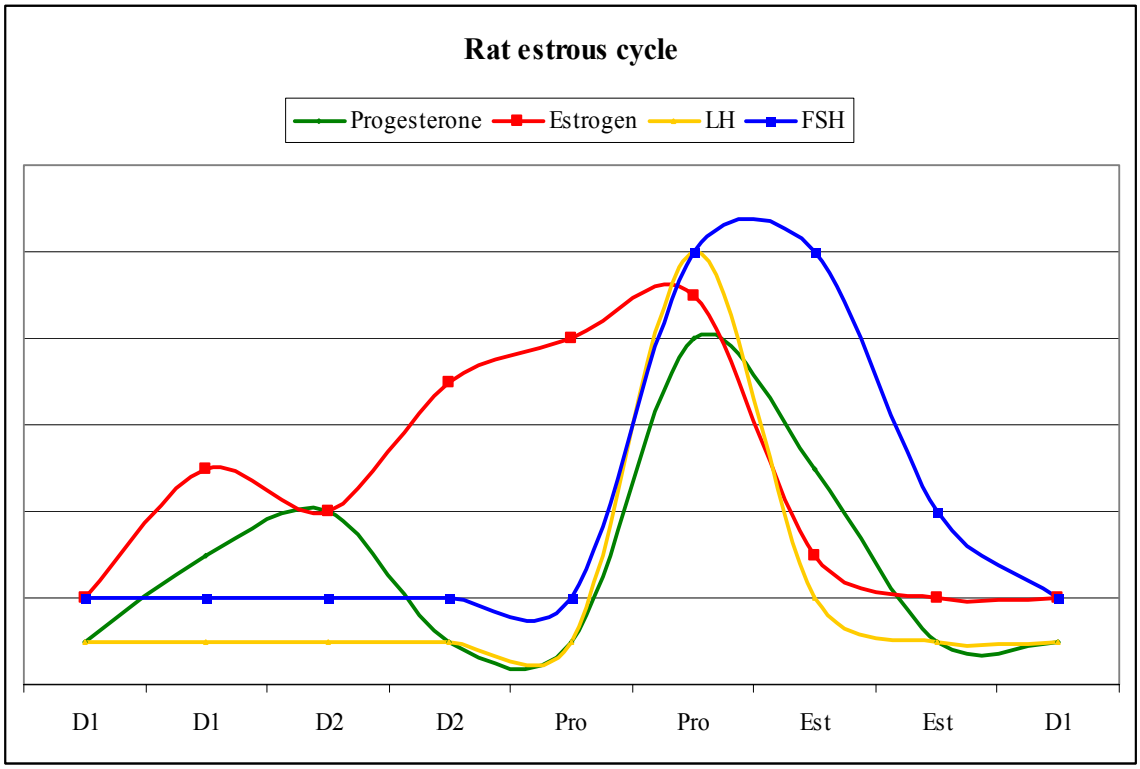


Fig 17 Hormone levels throughout rat estrous cycle (Fig. according to Marcondes et al. 2002)

Most of the animals displayed regular cycles, but there were a number of animals with irregular cycles showing extended proestrus/ estrus stages or non-cyclic characteristics.

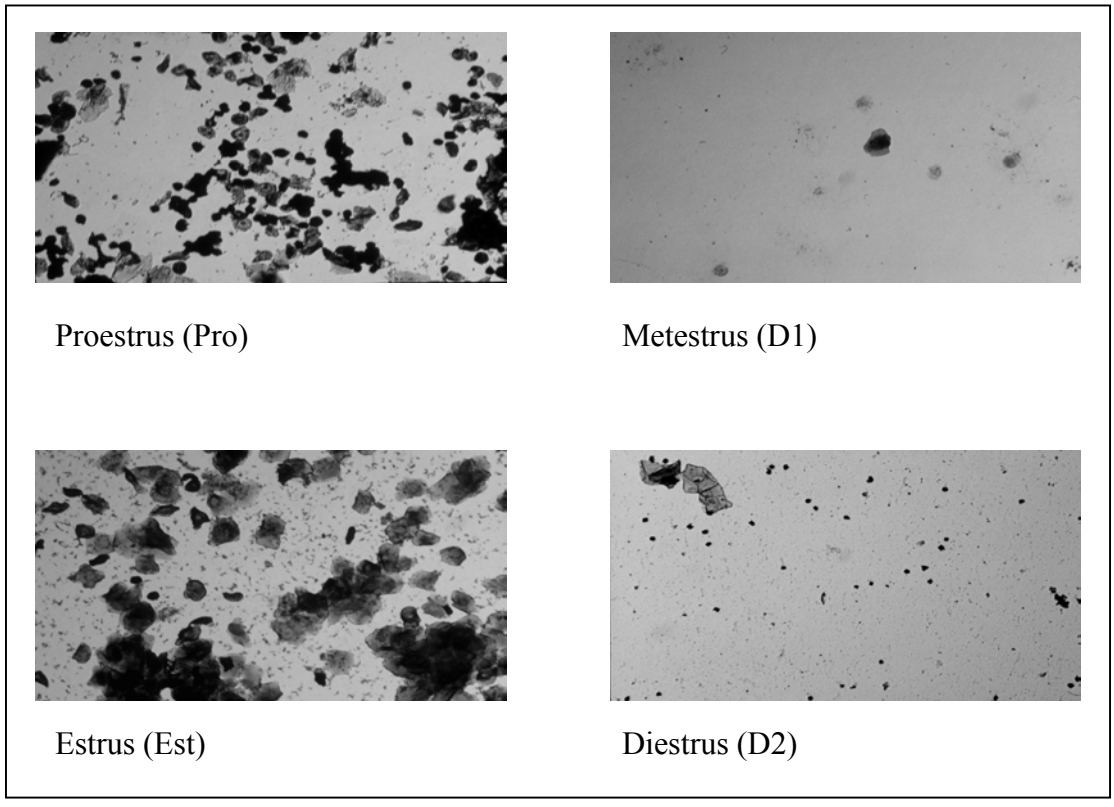


Fig. 18 Cell types throughout estrus cycle in Long Evan rats (see text for details)

Proestrus (Pro): The vaginal epithelium is thick and the follicles grow rapidly. The vaginal smear contains numerous small, nucleated epithelial cells, often in clusters and sometimes a few leucocytes.

Estrus (Est): The follicles grow to their maximum size. The smear contains large, anucleated cornified cells, partly in clusters. No leucocytes can be observed. In this state, progesterone is secreted from the corpus luteum. During early estrus, ovulation takes place.

Metestrus (Diestrus 1 = D1): In metestrus, leucocytes and very few small epithelial cells can be found in the smear. Here the levels of sex-hormones are low.

Diestrus (Diestrus 2 = D2): In this state of the estrous cycle, the follicles grow continuously. Numerous leucocytes and some small epithelial cells are to be found in the smear. In the Long Evans strain used in the present study, leucocyte number reached its maximum on D2.

E. Behavioural experiments

To test whether the changes in gene expression levels in brain regions (see chapter "Results") may have consequences at the functional level, a total number of 9 females each for controls and the high dose group of PBDE99 (P10) were tested for sexual behaviour.

At usual mating time, at around 4:00 pm, a normal, vigorous male taken from the pool of breeding animals was mated with either a control female or a P10-treated female in a cage (type 4), which is identical to the regular housing cage of the animals. The cover metal grid was removed, and replaced by a plexiglas attachment in order to heighten the walls to prevent the animals from escape. The video camera was positioned vertically above the cage. The animal room was illuminated by an infrared light source (Panasonic CCTV Camera, Model: WV-BP310/G) in order to record the activities with a special infrared camera (Fig. 19). Each experiment was recorded for 30 minutes in the late afternoon. All the females were tested with untreated males, chosen randomly from the pool of breeding animals, to assure, that experienced males were taken. Between subsequent experiments, the males were given a pause of at least four days to recover. Females were tested in proestrus. At around noon,

vaginal smears were taken to assess the stage of estrous cycle based on cytology (Cooper et al., 1993).

Observation Equipment

Fig. 19 *behavioural experiment's set up: observation chamber (1), infrared camera (2), red light (3), video recorder (4) and monitor (5)*

Determination of behavioural parameters

The videotapes were evaluated by counting the occurrence of the behavioural parameters listed below. In order to standardize the procedure of observation, it was carried out on coded tapes, without knowing the treatment group of the observed animal.

The following behaviours were evaluated:

LME: Lordosis with ejaculation

The male mounts the female, which displays lordosis. Successful intromission and ejaculation occurs. Indication for a successful performance is licking and cleaning activity of the male immediately following the ejaculation.

LOE: Lordosis without ejaculation

Male mounts the female, the female is displaying lordosis but no intromission occurs. No genital licking by the male.

MOL: mounting without Lordosis

Male mounting the female, but this does not display lordosis. No intromission occurs.

J&W: Jumping and wiggling

Female moves repeatedly in short jumps in front of the male and wiggles her ears. Characteristic for that behaviour is the straight tail and bent ears of the female.

LH: Licking head

Female licks or sniffs head or neck of the male.

LG: Licking genitals

Female licks or sniffs male's genitals.

F. Molecular biological methods

1. Determination of mRNA levels of estrogen regulated genes in two sexually dimorphic brain regions:

The F1-animals were sacrificed by decapitation by an animal guillotine in short-term ether anesthesia. Females of the steady state series were taken in Diestrus 1 as described above.

The brain was quickly removed, frozen on dry ice and stored at –80°C until molecular biological analysis. Cross sections of 300 µm thicknesses were cut in a Leitz 1720 cryostat equipped with a special knife, at the level of the medial preoptic nucleus (MPO) and of the ventromedial hypothalamic nucleus (VMH). The anterior-posterior development of the commisura anterior and the evolving 3rd ventricle were used for orientation. From these tissue slices, pieces containing MPO were cut with a scalpel, those containing the VMH with a preparation needle. (Fig. 22)

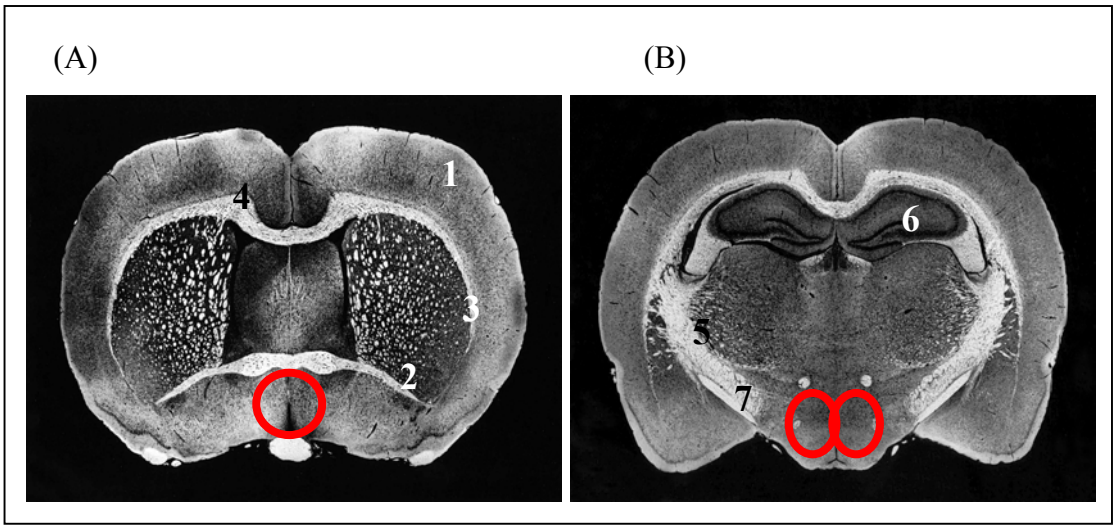


***Fig. 22**: Frontal brain slides with MPO (A) and VMH (B) (4,84x) 1: Corpus; 2: Commissura anterior; 3: nucleus caudatus putamen; 4: Radiatio corporis callosi; 5: capsula interna; 6: fimbria hippocampi; 7: tractus opticus*

2. RNA Isolation

The tissue samples were homogenized in RLT Lysis Buffer from QIAGEN (Basel, Switzerland) (5mg tissue/350µl Lysis Buffer), in a Polytron-rotor.
Total RNA was isolated following the procedure and using the reagents of the RNeasy mini Kit 250 (cat. No. 74106, QUIAGEN), plus a DNAse step.
For the reverse-transcription an RT-kit from Applied Biosystems (Rotkreuz, Switzerland) was used.

Homogenisation of the brain tissue

Brain pieces containing either MPO or VMH were put into Eppendorf tubes containing RLT buffer, 10µl ß-mercaptoethanol per 1 ml buffer. Tissues were homogenized in a rotor-stator homogenizer (Polytron, Kinematica, Lucerne, Switzerland) for 30 sec at room temperature and immediately centrifuged for 10 min at 3000 x g. The clear supernatant was stored at – 80°C. Between subsequent homogenisations the homogenizer was washed twice with water and one time with RLT-buffer.

RNA-Extraction

Total tissue ribonucleic acid (RNA) was extracted with the QIAGEN RNeasy-mini kit. After thawing the frozen probes they were centrifuged for 2 minutes at maximum speed (13000 rpm) to ensure that only clear solution was taken for the ongoing procedure. A total of 350 µl clear homogenate was added to 350 µl of 70 % ethanol and mixed carefully by pipetting up and down.

The 700 µl sample was put on a RNeasy mini column placed in a 2 ml collection tube. After 15 sec of centrifugation at 13000 rpm the eluate was discarded.
350 µl RW1 buffer was added to the centre of the column and the column was centrifuged for 15 sec. at 13000 rpm. The eluate again was discarded.
In order to quantify only mRNA by reverse transcription polymerase chain reaction (RT-PCR) any genomic desoxyribonucleic acid (DNA) must be quantitatively removed. Therefore the RNA samples were carried through a DNA-digesting step with RNase-free DNase 1-set (QIAGEN). DNase 1 was dissolved in 500 µl RNase-free water and then gently mixed by pipetting up and down. 20 µl aliquots were kept

at -20°C. Before use, 140 µl RDD buffer was added to one 20µl aliquot. Each RNA sample was incubated at room temperature for 15 min together with 80 µl DNase 1 preparation. After that, 350 µl RW 1 buffer was added, and the probe was centrifuged for 15 sec at 13000 rpm. The collection tube including the eluate was discarded; the column was transferred to a new 2 ml collection tube. 500 µl of RPE buffer, mixed with ethanol, was pipetted onto the column and centrifuged for 15 sec as described above. The eluate was discarded. Once again 500 µl RPE buffer was added on top of the column and centrifuged for 2 minutes at maximum speed.
For elution of total RNA, the RNeasy column was transferred to a new 1.5 ml collection tube and 30 µl RNase free water was added on top of the column. The column was centrifuged for 1 min at 13000 rpm and the sample of total RNA in the collection tube was immediately stored at -80 °C until use.

3. Reverse Transcription

To get a template for the final real time PCR procedure, and because it is more stabile than RNA, the RNA samples are reverse transcribed into cDNA. First, the master mix was prepared according to the table below. Then 20 µl RNA sample was added to each tube containing 80 µl of this mastermix. The samples were placed in a PTC-100 Programmable Thermal Controller (M Research Inc.), and the RT PCR reaction carried out under the conditions described in table 2.
As a result the complete set of RNAs in the two brain regions has been copied to cDNA. The samples were stored at –20°C until use for real time PCR.

Mastermix	Total volume 100ml	Final concentration
H_2O	28.5	
10x TaqMan RT buffer	10	1x
25mM $MgCl_2$	22	5.5mM
Deoxy NTP mix	20	500mM of each NTP
Random Hexamers	5	2.5mM
RNAse Inhibitor	2	0.4U/ml
MultiScribe TM RT	2.5	1.25U/ml
RNA in H_2O	10	1pg-20ng/ml

RT Conditions			
incubation	RT	RT inactivation	end
10 min	30 min	5 min	...
25 °C	48°C	95°C	4°C

Tab. 3 Master mix ingredients and conditions for reverse transcription

4. real time PCR

real time PCR is a skilful technique to quantitatively analyse the transcribed cDNA. Quantification is carried out by amplifying specific nucleic acid sequences using specific oligonucleotide primers and a DNA polymerase enzyme. This method does not require any post-PCR processing such as gel electrophoresis, for the quantification of the amplified product can be done at any point during the PCR cycles. Like in normal PCR, gene-specific forward and reverse primers are used. In addition, in real time PCR another specific oligonucleotide is introduced, which is labelled at its 3' and 5' end with a reporter fluorophor (FAM) and a quencher fluorophor (TAMRA), respectively. The quencher suppresses the reporter signal as long as it is located next to it. This TaqMan probe is designed to hybridise at the amplicon site between the two specific primers. During the amplification process the exonuclease activity of the polymerase enzyme leads to a cleavage of the TaqMan probe and the reporter fluorophor is spatially separated from the quencher. Thus the quencher fluorophor no longer suppresses the fluorescent signal from the reporter and the emitted fluorescence can be detected. A laser is used to excite the electrons from the fluorescein molecules (Fig. 23)

The real time PCR reaction and the resulting amplification plot have a sigmoidal shape. Three different phases can be observed: a background phase at which the fluorescence signal is masked by the background signal of either machine or reaction, an exponential phase in which the amount of cDNA is doubling within each cycle of the ongoing reaction, and a plateau phase. During the background phase, the signal of the PCR product is below the background signal. As soon as a sufficient amount of product has been amplified and can be detected, the signal exceeds the background and the exponential phase starts. In this phase the amount of cDNA doubles within one cycle. The reaction is finished, when no further amplification takes place. Then the plateau phase, in which the slope of the curve is tending towards zero, is reached.

The Ct value (**t**hreshold **c**ycle) is defined as the point where the signal of the reporter exceeds the background. This is also the starting point of the exponential phase. With this Ct value the exact amount of product (this is the amount of gene specific cDNA) at the beginning of the amplification can be calculated. The lower the Ct-value is, the higher the initial amount of cDNA was.

In order to balance different efficiencies in the RNA extraction step, the level of mRNA of the gene of interest is normalized to the amount of a housekeeping gene, which is stable and remains largely unchanged by chemical treatment. Thus the housekeeping gene serves as an internal reference.

For real time PCR quantification, the ABI PRISM™ 7700 Sequence Detector (Applied Biosystems) was used, which consists of a 96-well thermocycler, argon laser and CCD camera. Samples were analysed with the ABI 7700 Sequence Detector software (Lie et al. 1998).

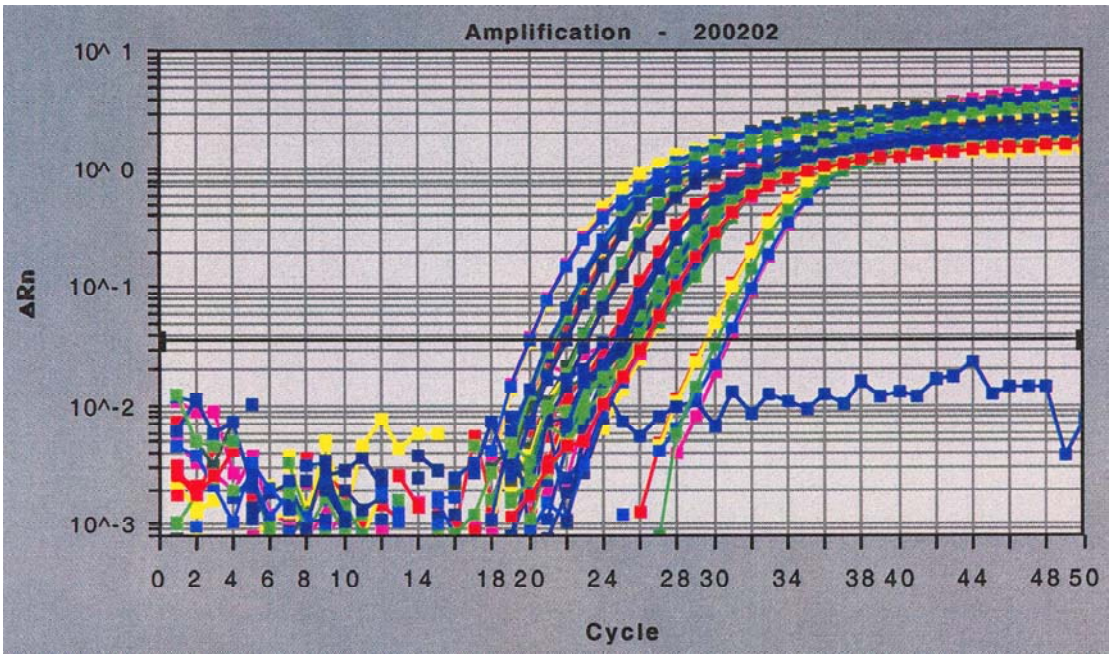


Fig. 23 *real time PCR plot with exponential curves of cDNA amplification with background phase (cycles 0 to 16), exponential phase (cycles 16 to30) and plateau phase (cycles 30 to 50)*

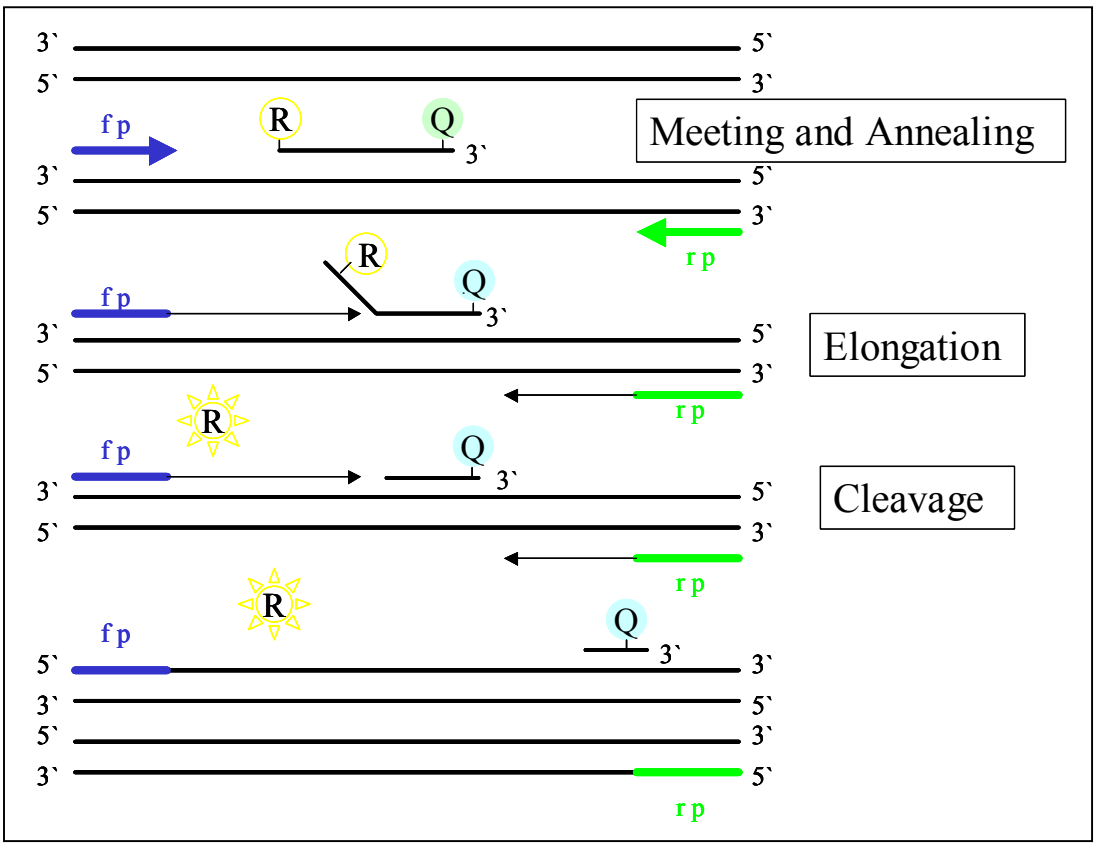


Fig. 24 During one amplification cycle of PCR forward (fp) and reverse primers (rp) hybridize to the fitting sequence of the target DNA. The TaqMan probe hybridizes in between of them. With its 5'-nuclease property the Polymerase enzyme cleaves the TaqMan probe thus the reporter dye (R) and quencher dye (Q) are being separated, resulting in fluorescence of the reporter. (R).

Master Mix	µl	Final concentration
TaqMan Universal PCR Master Mix (2x)	12.5	-
Forward Primer	2.5	300 nM
Reverse Primer	2.5	300 nM
TaqMan probe	2.5	125-150 nM
DNA sample	2.5	-
H_2O	2.5	-
Total	25	-

Tab. 4 real time PCR Master Mix agents

Procedure

For the analysis a 25 µl reaction volume was used, consisting of 2.5 µl of each cDNA sample and 22.5 µl Mastermix, containing H_2O, forward and reverse primer (300 nM each) and TaqMan probe (225 nM) (Table 4). The real time PCR reactions were carried out in 96-well reaction plates with optical caps (Applied Biosystems). The arrangement of samples on the plates is shown in Fig. 25. For calculation a standard curve was generated with four concentration points, pipetted in triplicates for the gene of interest, and another one of the same type for the reference gene cyclophilin A. The 72 wells left were filled with the samples according to the scheme given in Fig. 25

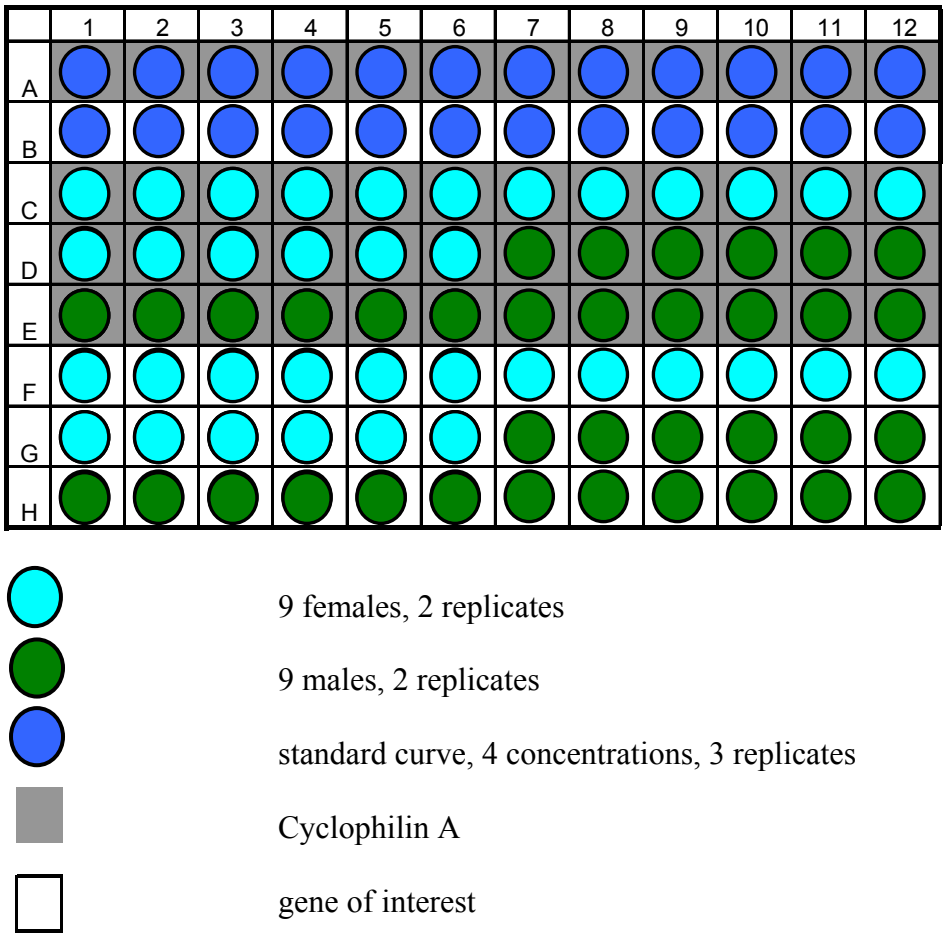


Fig. 25 *optical plate with 96 wells: arrangement for comparison of gene expression in males and females*

This way it was possible to test 18 samples within one run on one plate. To be able to detect possible sex differences I decided to put samples from 9 males and 9 females of one treatment group and one brain region onto one plate. The real time PCR reaction was then performed on the ABI PRISM 7700 real time thermocycler (Applied Biosystems). PCRs were started at a hold temperature of 50°C for 2 minutes, followed by 95°C for 10 minutes. Then the reaction underwent 40 cycles, each with 95°C for 15 s followed by 60°C for 60 s.

real time data analysis

Results were analyzed using Sequence Detector Software SDS 2.0 (Applied Biosystems). When the reaction was finished, the threshold line was manually set in the linear region of the curves above the background reaction, and the threshold cycle (Ct) was defined as the cycle number at which the threshold line crosses the amplification plot. Further analyses were then done in Microsoft Excel®. As the amplification efficiencies of the reference gene and the target gene were not equal, as revealed by different slopes of the exponential phase and different maxima for fluorescence in real time PCR, the standard curve method was chosen. To obtain standard curve plots cDNA of an RT sample of total brain from control animals was plotted for either gene on a 96 well plate taking 4 measurement points with triple replicates each and a dilution factor of 1:2.

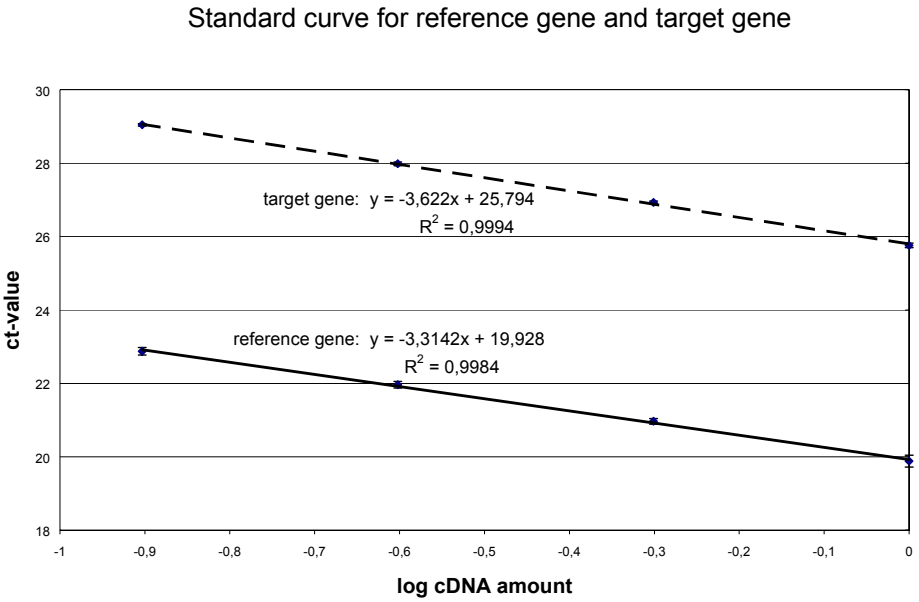


Fig. 26 Example of a standard curve of a reference gene (cyclophilin) and a target gene

The general standard curve formula is y = mx + q. Y represents the Ct-value, m the slope of the standard curve line, x stands for log cDNA amount, q for the y-intercept of the standard curve line. The cDNA amount is calculated as 10^x, where x means (y-q) / m

To quantify the cDNA amount of the target gene, the target gene reading is normalized to that of the reference gene cyclophilin. i.e., the target gene cDNA amount is divided by the cyclophilin cDNA amount. cDNA amounts of target gene

and cyclophilin, are calculated from the Ct-value of each experimental sample using the standard curves for both genes of the particular run.
Values of mRNA shown for a target gene in a given tissue thus represent values relative to the amount of the reference gene.

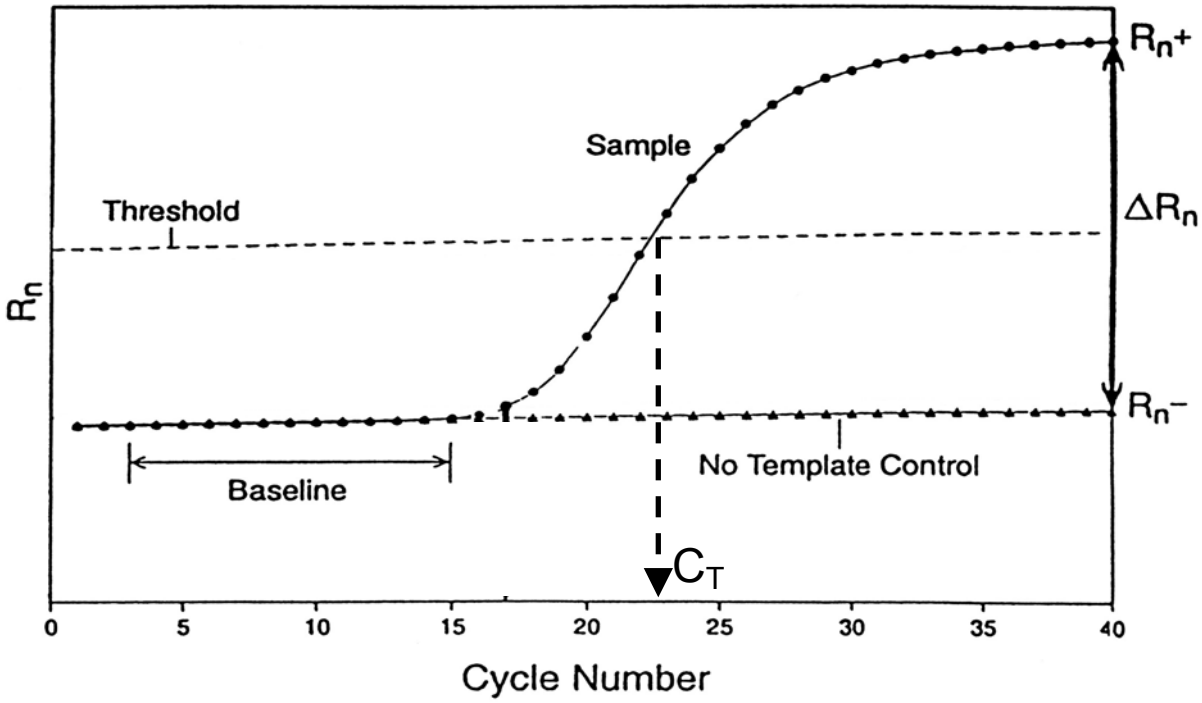


Fig. 27 real time PCR curve shape: the real time PCR reaction basically consists of three stages, an early baseline phase representing the background noise, an exponential phase and a plateau phase. The cycle in which the sample exceeds the threshold is called threshold cycle (C_T). The C_T value gives the exact amount of gene specific cDNA. The no template control is a sample, in which there is no cDNA. Therefore it never exceeds the baseline signal. Delta Rn is directly linked to the amount of fluorescence.

Primer and TaqMan probe design

The Gene Bank of NCBI organisation served as source for mRNA sequences for all genes (http://www.ncbi.nlm.nih.gov). Primers and TaqMan probes were designed with PrimerExpress Software, version: 2.0 (Applied Biosystems). All primers as well as the probes were synthesized by Microsynth (Balgach, Switzerland). TaqMan probes were labelled with FAM (6-carboxyfluorescein) at the 5´ end as the reporter and TAMRA (6-carboxytetramethylrhodamine) at their 3' end as the quencher. Primer and probe concentrations were optimized according to the instructions given by the company (ABI Prism "User Bulletin 2"). The design of primers and TaqMan probes was carried out according to the following criteria:

Primers: The G/C content spans a range of 30-80 %; the melting point is around 58-60°C; the nucleotide at its 3' end is G or C, the optimal length is about 20 bases.
TaqMan Probe: 5' end has to be C, A or T, no G; the number of C bases is higher than G bases; the amount of G and C bases together is around 30-80 % and the melting point is around 68-70°C.

Primer and probe sequences used for real time PCR:

Forward Primers (FP), Reverse Primers (RP), and TaqMan Probes are listed with the corresponding GenBank™ Accession Number for the following genes: Estrogene receptor (ER) alpha and beta, Progesterone receptor (PR), Preproenkephalin (PPE) and Cyclophilin (Cyc). The sequences of PR, ER and Cyc were kindly provided by coworkers from our laboratory.

Primer and probe sequences		
Estrogene Receptor alpha (NM012689)	FP:	5'-CCAAAGCCTCGGGAATGG-3'
	RP:	5'-AGCTGCGGGCGATTGAG-3'
	Probe:	5'-TCGTTCCCTTGGATCTGGTGCAACAA-3'
Estrogene Receptor beta (NM012754)	FP:	5'-TTGGTGTGAAGCAAGATCACTAGAG-3'
	RP:	5'-AACAGGGCTGGCACAACTG-3'
	Probe:	5'-CCACTAAGCTTCCTCTTCAGTGTCTCTCTGTTTACA-3'
Progesterone Receptor (NM022847)	FP:	5'-GCAATTGGCTTAAGACAGAAAGG-3'
	RP:	5'-ACAAGATCATGCAAGCTGTCAAG-3'
	Probe:	5'-CCCAGTTCACAACGCTTCTATCAACTTACAAAAC-3'
Preproenkephalin (M28263)	FP :	5`-AGCTACCTGCGCCATCTGAA-3`
	RP :	5´-CCAGATGCAAAGTCTCAGGAACT-3´
	Probe :	5´-AACGCAGCCCCATGGCG-3´
Cyclophillin A (M19533)	FP:	5'-TGTGCCAGGGTGGTGACTT-3'
	RP:	5'-TCAAATTTCTCTCCGTAGATGGACTT-3'
	Probe:	5'-CCACCAGTGCCATTATGGCGTGT-3'

Tab. 5 Accession numbers and sequences of primers and TaqMan probes of the different genes used for real time PCR (FP = forward primer, RP = reverse primer, Probe = TaqMan probe, A = Adenine, C = Cytosine, G = Guanine, T = Thymine, 5´ = 5´-end, 3´ = 3´-end)

	Steady state condition		Acute estrogen challenge	
	VMH	MPO	VMH	MPO
PR	x	x	x	x
ER alpha	x	x	-	-
ER beta	X	x	-	-
PPE	x	x	x	x

Tab. 6 synopsis of mRNA species analyzed in different parts of the project:

G. Tissue levels of PBDE99

In order to determine the tissue levels achieved by the treatments samples were analyzed (by EUKOS, Plön, Germany). The samples were worked off in this laboratory in a 7+2-mixture of hexane/isopropanol. The extract then was analyzed by capillary gas chromatography with mass spectrometry in single ion detection mode (GC/MS-SIM).

H. Statistical analysis

Data from the different treatment groups, including the vehicle control, were analysed using analysis of variance (ANOVA) followed by pairwise comparison with Bonferroni correction (SYSTAT software version 5.02). Significance (with $p < 0,05$) is indicated by asterisks.

I. Instruments and Chemicals

Instruments

Infrared Camera:	Panasonic CCTV Camera, Model: WV-BP310/G
Microcentrifuge:	Eppendorf Cenrtifuge 5415D
Reverse Transcription:	PTC-1000
Realtime PCR:	ABI Prism 7700 Sequence Detector
Videotapes:	VHS Broadcast Master E180
Homogenizer:	Polytron, Kinematica, Lucerne, Switzerland

Chemicals and disposables

Aroclor1254	commercial PCB-mixture (Promochem, Wesel, Germany)
PBDE99	2,2',4,4',5-Pentabromodiphenylether, purity > 99% (ditto)
Taq Man	Taq Man Universal PCR Master Mix Roche
Dnase	Rnase free Dnase Set QIAGEN
RLT buffer	Lysis buffer QIAGEN
EtOH	Fluka
Pipette tips	Biosphere Filter Tips (RNase free), Sarstedt
Tubes	Sarstedt
RDD buffer	Rnase free Dnase set QIAGEN
Rt PCR wells	96 well optical plates, Applied Biosystems (AB)
RT Set	Taq Man® Reverse transcription Reagents (AB)
RW1 buffer	wash buffer QIAGEN
RPE buffer	220 ml RPE buffer (QIAGEN) + 55 ml EtOH 100%

RESULTS

To investigate the developmental neuroendocrine toxicity of PBDE99 at functional and molecular levels PBDE 99 (2,2',4,4',5-PentaBDE, 1 or 10 mg/kg/day) was administered s.c. to time-pregnant Long Evans rats from gestational day 10 to 18 (vehicle control olive oil). Several developmental landmarks have been studied and mRNA levels of several sex hormone-regulated genes were quantified by real time PCR (cyclophilin as reference gene) in two sexually dimorphic brain regions, involved in control of sexual behavior and gonadal regulation, ventromedial hypothalamic nucleus (VMH) and medial preoptic region (MPO). Prenatal exposure to PBDE99 was studied first under steady state conditions and in a subsequent experiment with an acute challenge setup. Finally female sexual behavior was studied in the 10 mg/kg PBDE99 group. Table 1 shows the total number of tested rat F1 offspring.

Treatment group	Number of females	Number of males	Number of litters
Controls (C)	18	20	9
PBDE99 1mg/kg (P1)	12	14	7
PBDE99 10mg/kg (P10)	10	22	9
Aroclor1254 10mg/kg (Aro10)	13	20	5
Aroclor1254 30mg/kg (Aro30)	6	12	4

Tab. 7 number of animals and number of litters

A. General toxicity data

1. litter size and sex ratio

The slight changes amongst the different treatment groups in litter size as well as in the sexratio appeared not to be significant.

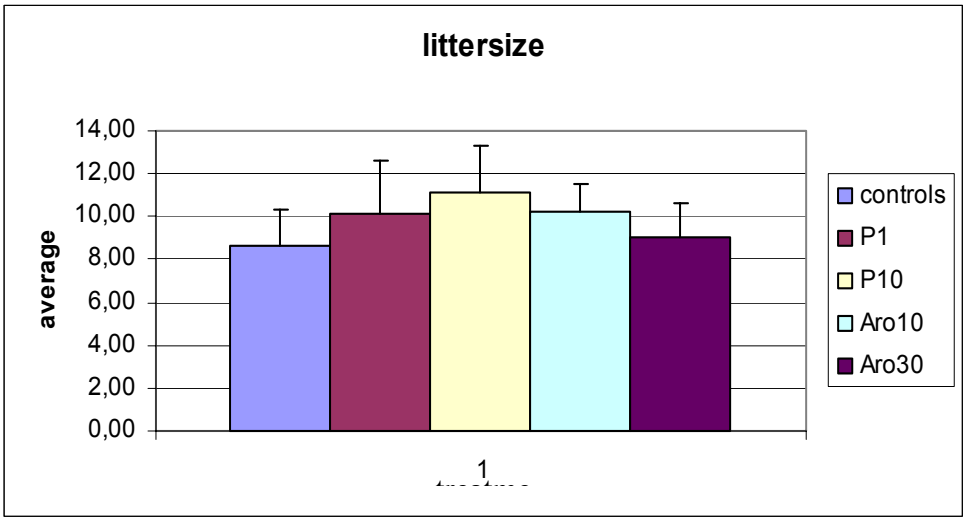


Fig 28 littersize of the different treatment groups

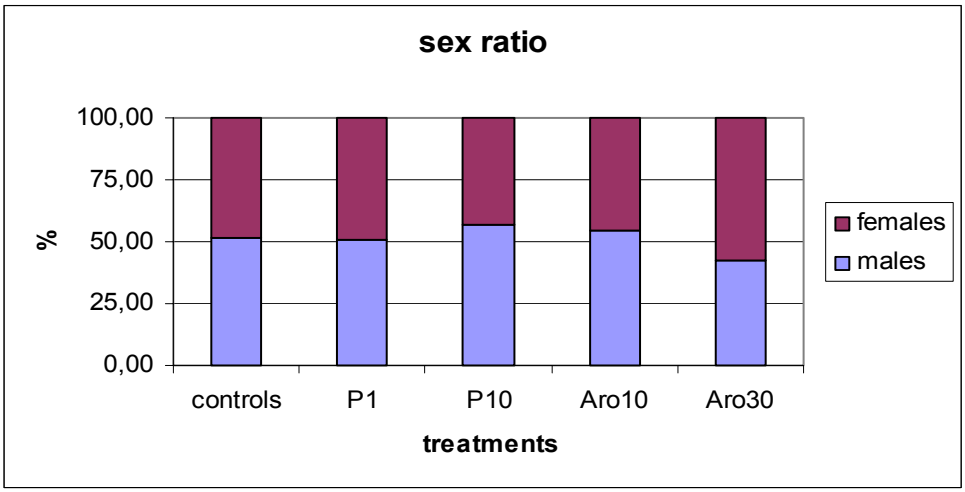


Fig. 29 sex ratio of the different treatment groups

2. Survival rate

Except for 30mg/kg of Aroclor 1254, the chemicals did not affect the survival rate of prenatally treated offspring in the dose range examined. This indicates that there are no general toxic effects of the substances tested. In the Aro30 group, the survival rate appeared to be reduced but this effect was not statistically significant, possibly because of the small litter number. Since later, at an age of about 5 weeks, some of the exposed female offspring suddenly died, the experiments with this dose were discontinued.

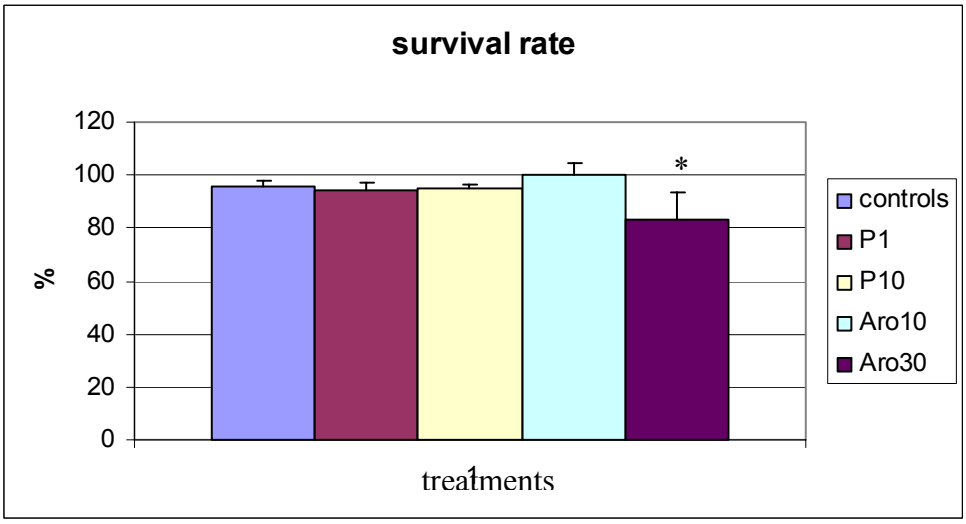


Fig. 30 survival rate of all offspring surviving at least until PN14 as percentage of alive born animals. Controls (C), PBDE99 1mg/kg/d (P1), PBDE99 10mg/kg/d (P10), Aroclor1254 10mg/kg/d (Aro10) and Aroclor1254 30mg/kg/d (Aro30). Mean values +/- S.E.M., n see table 7

3. Body weight

Adult body weight also serves as a parameter for the detection of possible general toxic effects.

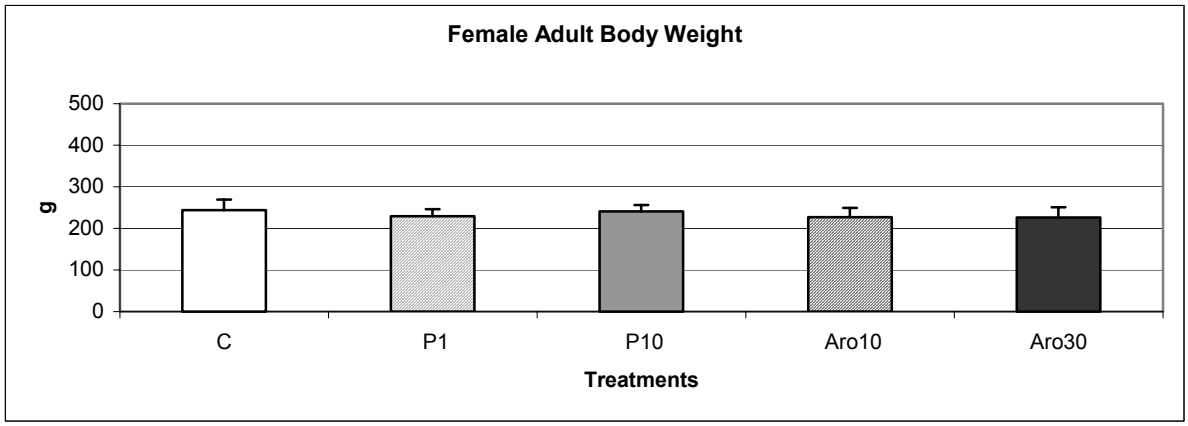


Fig. 31 Body weights of adult female offspring in different treatment groups. Mean +/- S.E.M., for n see table 7

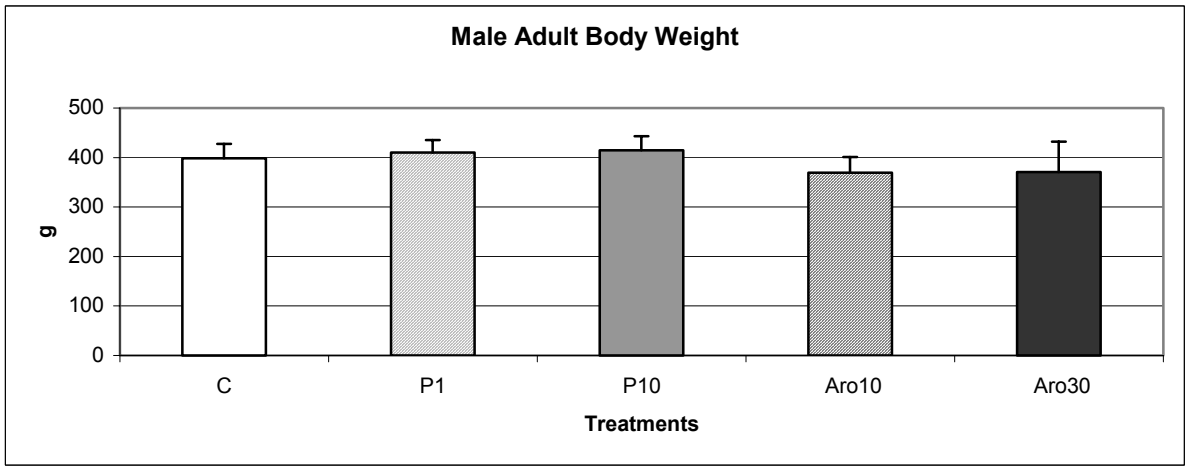


Fig. 32 Body weights of adult male offspring in different treatment groups. Mean +/- SEM, for n see table 7

Figures 31 and 32 show that also from the point of view of body weight in early adulthood there is no evidence for general toxicity in adult offspring.

4. Adipose tissue levels

Treatment with 1 or 10 mg/kg resulted in the following PBDE99-levels in rat offspring (analyzed by EUKOS, Plön, Germany):

Neonatal brain 0.04 +/- 0.02 µg/g lipid (n=2), and 0.21 +/- 0.006 µg/g lipid (pooled brains). Adipose tissue of 120 day-old adult offspring 0.42 +/- 0.14 µg/g lipid (n=2), and 7.4 +/- 3.0 µg/g lipid (n=11) (males + females combined).

B. Endocrine development

1. Anogenital distance

Absolute as well as relative anogenital distance exhibited no significant changes in different treatment groups.

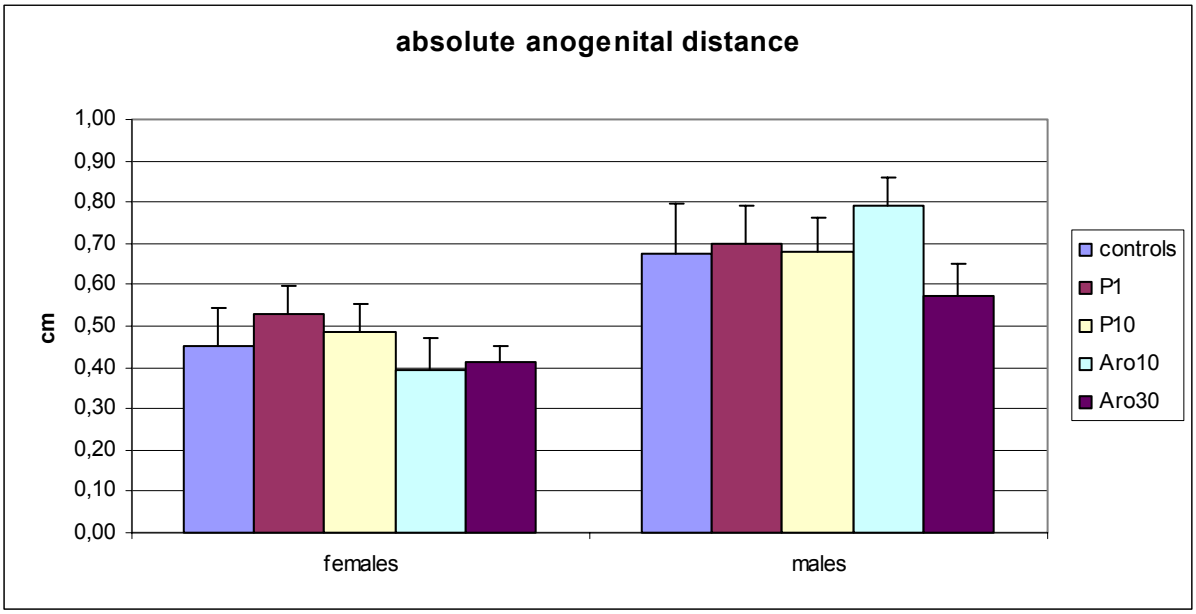


Fig. 33 Absolute anogenital distance of offspring in different treatment groups. Mean +/- SEM, for n see table 7

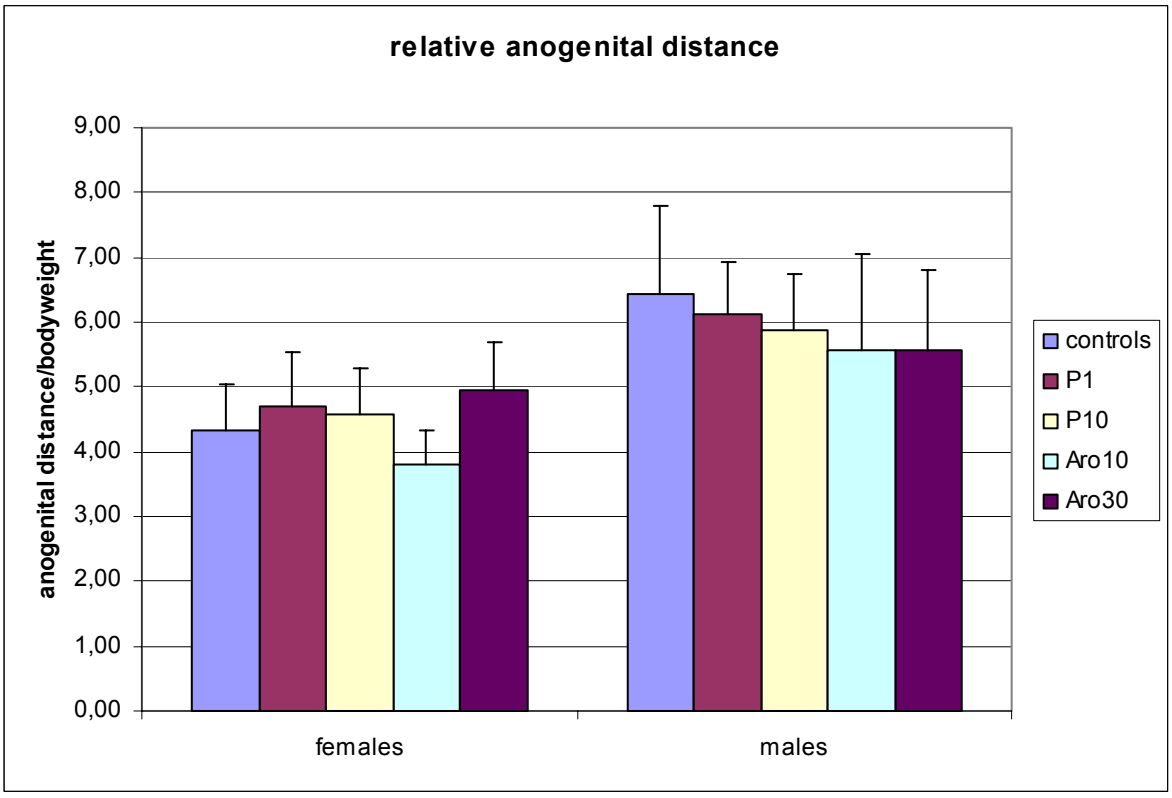


Fig. 34 Relative anogenital distance of offspring in different treatment groups. Mean +/- SEM, for n see table 7

2. Reflexes (righting)

Offspring were positioned on their back to test for reflexes. When they were turning back within 6 seconds they were considered as positive. The Aro30-treatment tended to reduce this reaction.

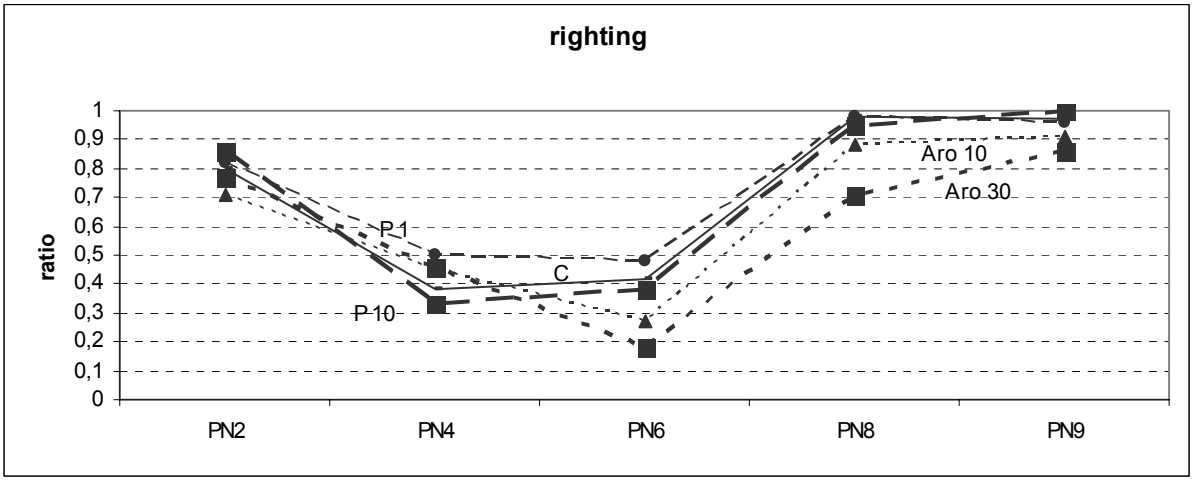


Fig 35 righting behavior of offspring in different treatment groups. Ratio positive animals, for n see table 7

3. Onset of puberty

Prenatal treatment with both, PBDE99 (10 mg/kg/day) and Aroclor 1254 (30 mg/kg/day) significantly ($p < 0.05$) delayed the onset of puberty in female offspring (table 8). Male puberty was slightly but significantly ($p < 0.05$) advanced by Aroclor (10 mg/kg/day), and a similar tendency was noted with 1 mg/kg/day PBDE99 ($p < 0.05$) and 10 mg/kg/day PBDE99 ($p < 0.01$), (table 9).

Vaginal opening (VO)

females	aver. PN	Stdev.	aver. weight	stdev.
C	39,55	1,33	116,43	7,55
Aro 10	39,13	1,41	104,65	20,76
Aro 30	41,92	2,75	73,30	26,90
P 1	40,16	1,91	122,17	6,79
P 10	40,89	2,67	123,35	9,86

Tab. 8 Onset of puberty (females) and bodyweight at vaginal opening

preputial separation (PS)

males	aver. PN	stdev.	aver. weight	stdev.
C	47,64	1,17	182,34	12,36
Aro 10	46,72	1,45	171,99	21,34
Aro 30	48,00	2,00	160,99	13,01
P 1	46,62	1,32	172,53	15,46
P 10	46,33	1,35	172,50	14,76

Tab. 9 Onset of puberty (males) and bodyweight at preputial separation

4. Eyes opening

At PN14 the opening of the eyes was examined. The low dose of PBDE99 happened to delay, whereas the high dose of Aroclor1254 advanced that developmental parameter. The other treatments did not have a significant effect.

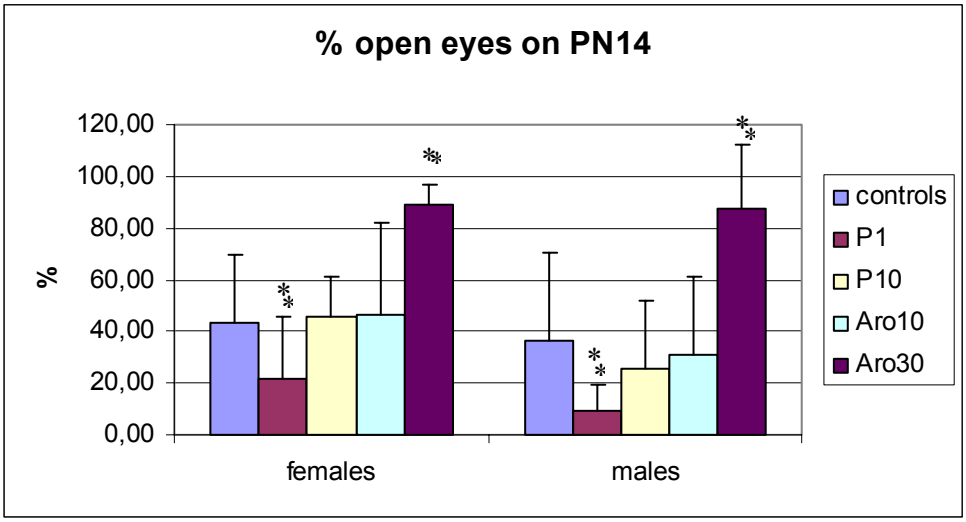


Fig. 36 Ratio of offspring with open eyes at PN14 in different treatment groups. Mean +/- SEM, for n see table 7. Significant changes are indicated by asterisks ($p < 0.05$)

C. Sexual functions in adult offspring

1. Estrous cycle in baseline F1 females

To detect a possible impact on the control of the gonadal axis in exposed F1 females, their estrous cycles were examined with regard to their regularity. Vaginal smears were taken for 21 days. Cycles exhibiting 4 or 5 days of recurrent cycles respectively

were classified as regular. Then the percentage of cycles with no detectable regularity were calculated. The results show that exposure to PBDE99 at both dosage levels, as well as exposure to the PCB-mixture Aroclor1254, had severe consequences. In all prenatally treated females the estrous cycle was extensively altered.

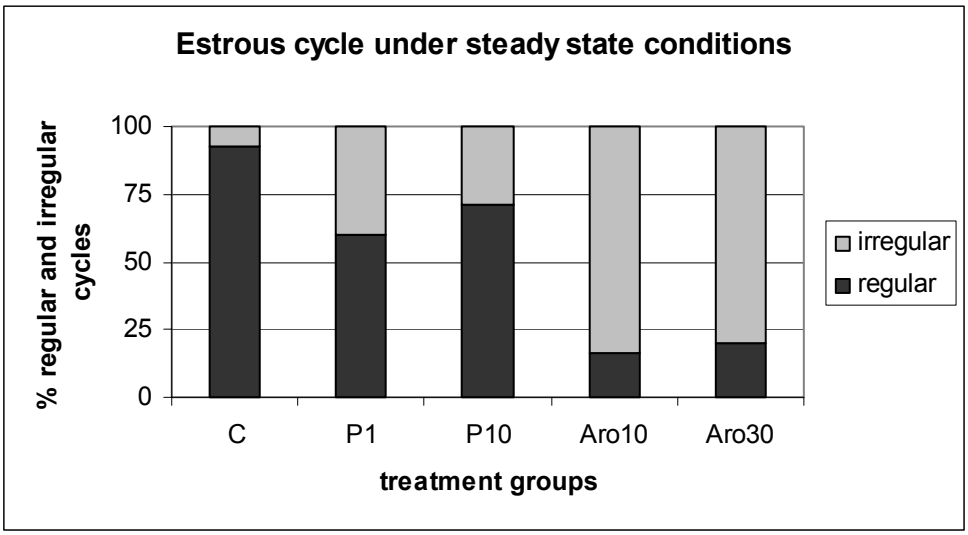


Fig. 37 Percentage of regular estrous cycles in different treatment groups. C = controls, P1 = PBDE99 1 mg/kg/d, P10 = PBDE99 10 mg/kg/d, Aro10 = Aroclor1254 10 mg/kg/d, Aro30 = Aroclor1254 30 mg/kg/d

2. Female sexual behaviour

Female sexual behaviour was analyzed in proestrus defined by vaginal smear. Females were mated with an untreated, sexually experienced male. Except for “licking head” and “licking genitals” behaviours (LH + LG), sexual behaviour was markedly altered by PBDE99 (Fig. 38). Female approaching behaviour towards the males, like jumping and wiggling, was dramatically reduced. Since the lordosis reaction, a receptive behaviour of the female, is independent of the male ejaculatory behaviour, the lordosis quotient (LQ) is calculated by dividing the number of behaviours, in which lordosis occurs (LME, LOE and MOL), by the total number of mounts. The lordosis quotient was significantly lower in PBDE99-exposed offspring as compared to the control group. Thus the evaluation of female sexual behaviour revealed PBDE99-induced changes in female sexual activity.

The total count of mounts, corresponding to LME + LOE + MOL also showed a significant decrease in the treated group, possibly as a result of reduced attraction by the female. Video recordings were taken during 30 minutes, and the following

parameters were analyzed on the video tapes: LH, LG, JW, MOL, LOE, LME (see chapter Materials and Methods).

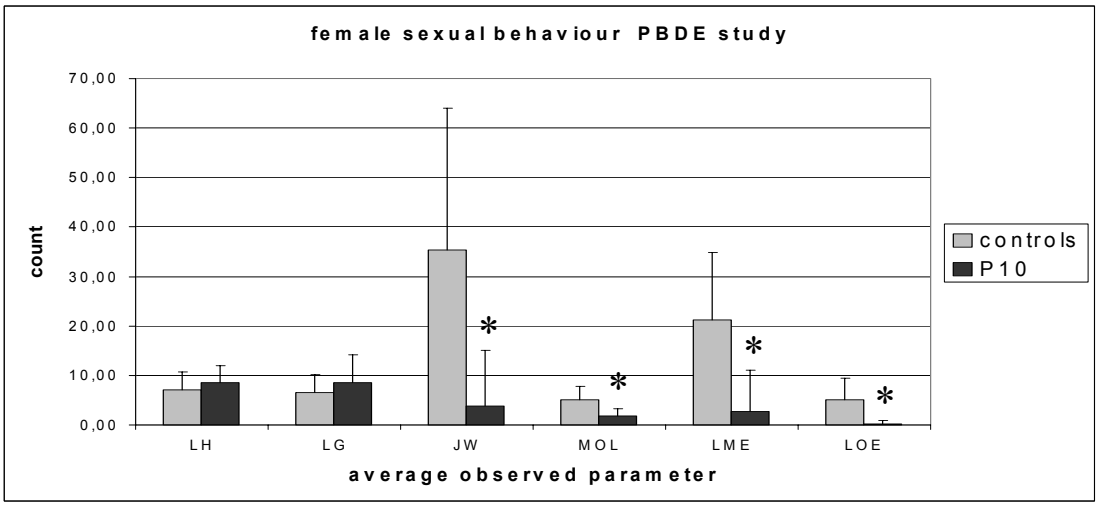


Fig 38 Female sexual behavior: the behaviors observed are LH = female licking or sniffing head of male, LG = female licking or sniffing genitals of male, JW = jumping and wiggling (approaching behaviour of female towards male), MOL = mounting without lordosis, LME = lordosis with ejaculation, LOE = lordosis without ejaculation; (Mean +/- SD, n = 9 for both groups) (* = $p < 0.01$ in ANOVA test with pairwise comparison and Bonferroni correction)

Lordosis Quotient

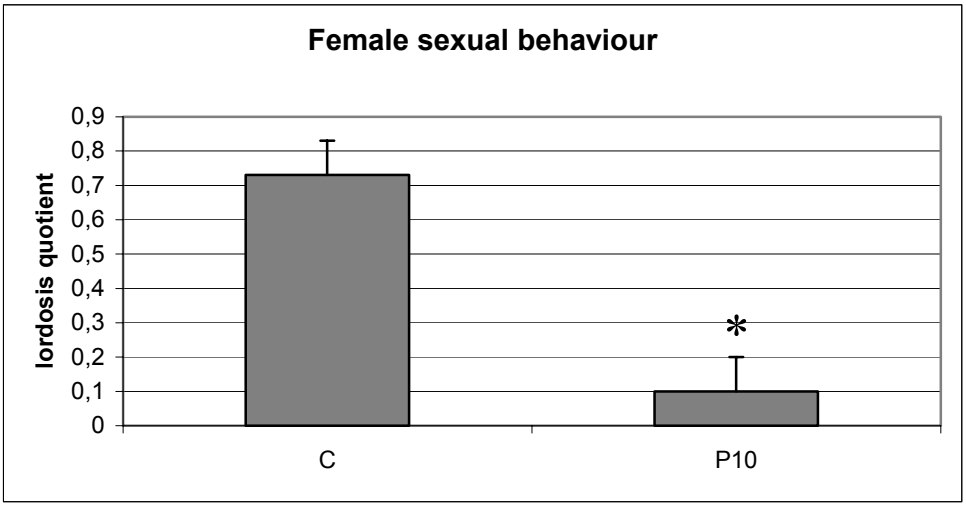


Fig 39 The Lordosis Quotient (lordosis behaviours / total number of mounts) as an indicator for female perceptive behaviour (n = 9 for both groups) (* = $p < 0.01$ in ANOVA test with pairwise comparison and Bonferroni correction)

D. Estrogen target gene mRNA expression under steady state conditions (baseline)

Results of the molecular biological analysis of adult F1 animals under steady state conditions are shown in this chapter. Females were studied in diestrus 1 (metestrus) which is characterized by low endogenous sex hormone levels. The test compounds were administered by s.c. injection to the pregnant dam from gestational day (GD) 10 to 18. Because the compounds bioaccumulate the offspring are exposed from late embryonic life until adulthood, including the period of sexual brain differentiation (from GD18 to postnatal day 7).

Ventromedial hypothalamic nucleus: mRNA expression in adult male and female F1 rat offspring - baseline series

mRNA levels were determined in a tissue piece containing the ventromedial hypothalamic nucleus (VMH) by real time RT PCR.

Progesterone receptor mRNA in VMH

Progesterone receptor (PR) mRNA exhibited a significant sex difference between males and females in the control group. The female PR mRNA level in the VMH is higher than in males. This sex difference was abolished in the treatment groups, both the PBDE99- and the PCB-groups, with the females showing a clear decrease of progesterone receptor mRNA. Male PR mRNA levels increased significantly in the low dose PBDE99 group (P1). In this treatment group, male and female levels were identical.

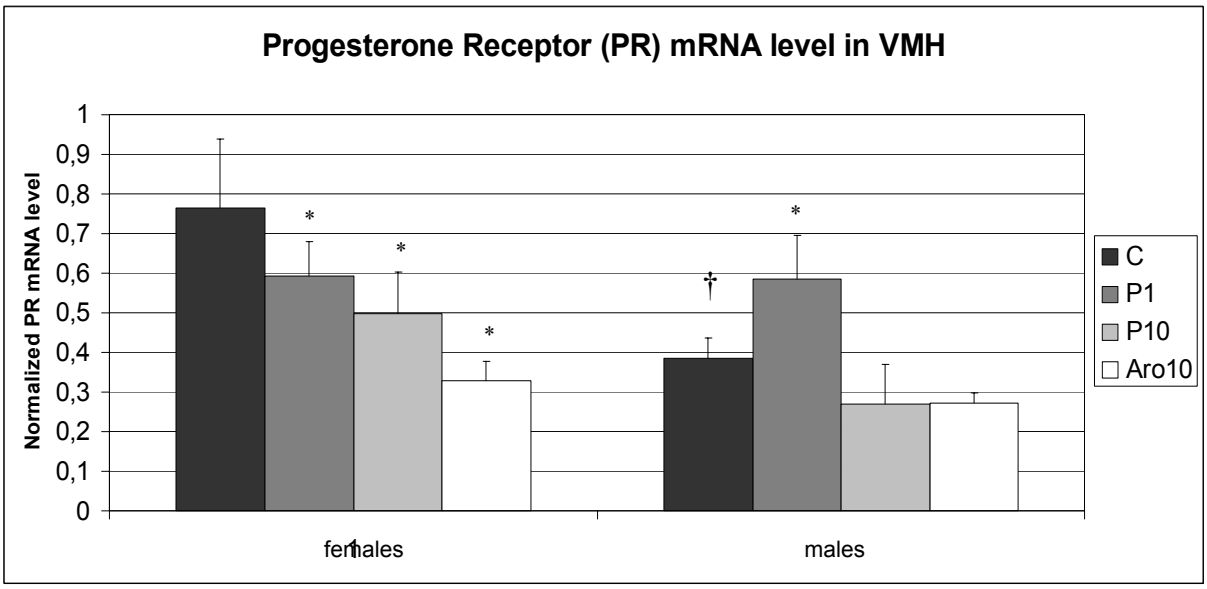


Fig. 40 Progesterone receptor (PR) mRNA level in ventromedial hypothalamus (VMH) of adult male and female F1 rat offspring. Mean ± S.D. (n=9). The level of progesterone receptor mRNA is normalized to cyclophilin mRNA (Cyc). Statistically significant differences of mRNA expression compared to control animals are indicated by * ($p<0.05$). † indicates a significant difference between control males and females ($p<0.05$).

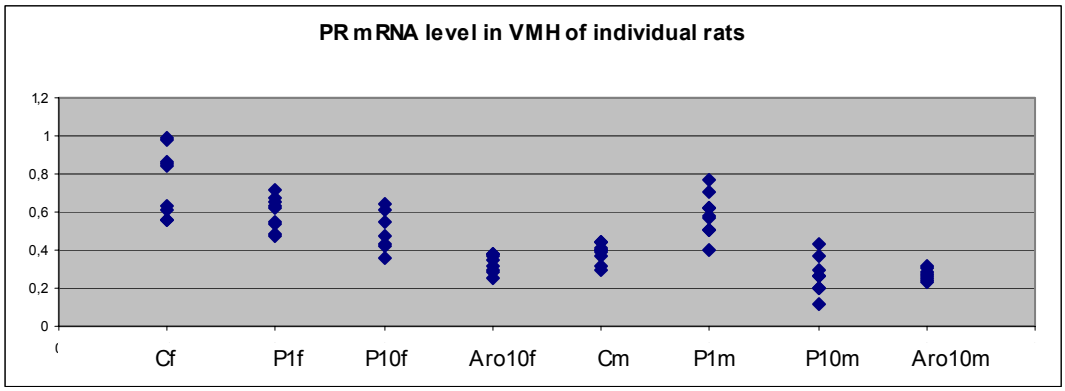


Fig. 41 Progesterone receptor mRNA (PR) level in the ventromedial hypothalamus (VMH) of individual animals. PR mRNA level normalized to cyclophilin (Cyc). (n= 9 per group).

Estrogen receptor alpha mRNA in VMH

Both treatments, PBDE as well as PCB, lead to a significant increase of ER alpha mRNA level. The females appeared to be more affected than the males, where the increase was by far less considerable.

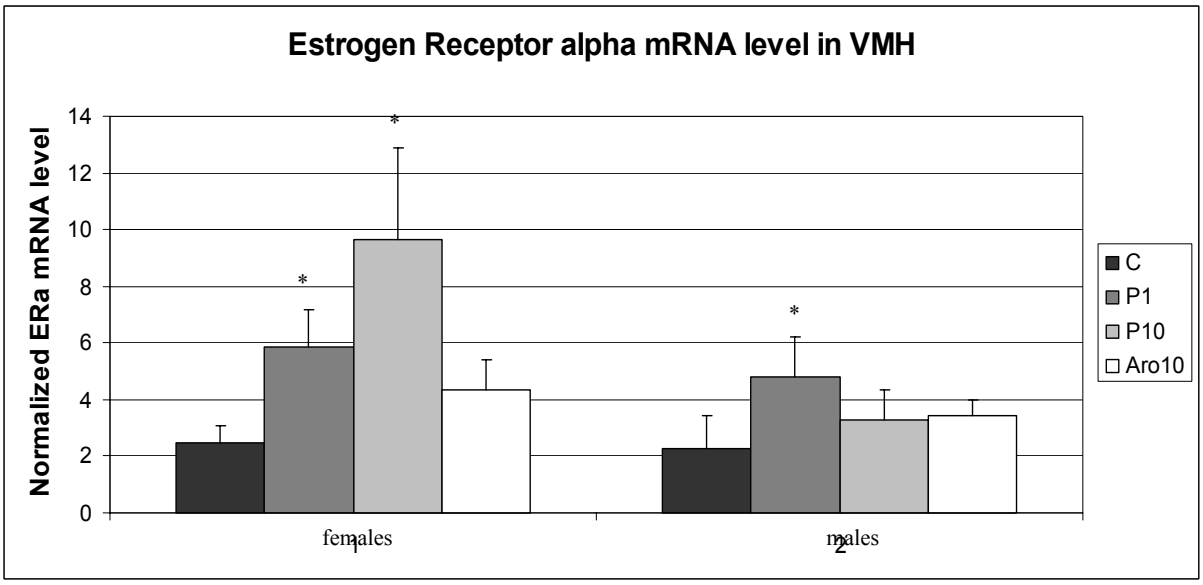


Fig. 42 Estrogen receptor alpha (ER alpha) mRNA level in the ventromedial hypothalamus (VMH) of adult male and female rat offspring. Mean ± S.D. (n=9) mRNA level of estrogen receptor alpha normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of mRNA level compared to control are indicated by * ($p<0.05$)

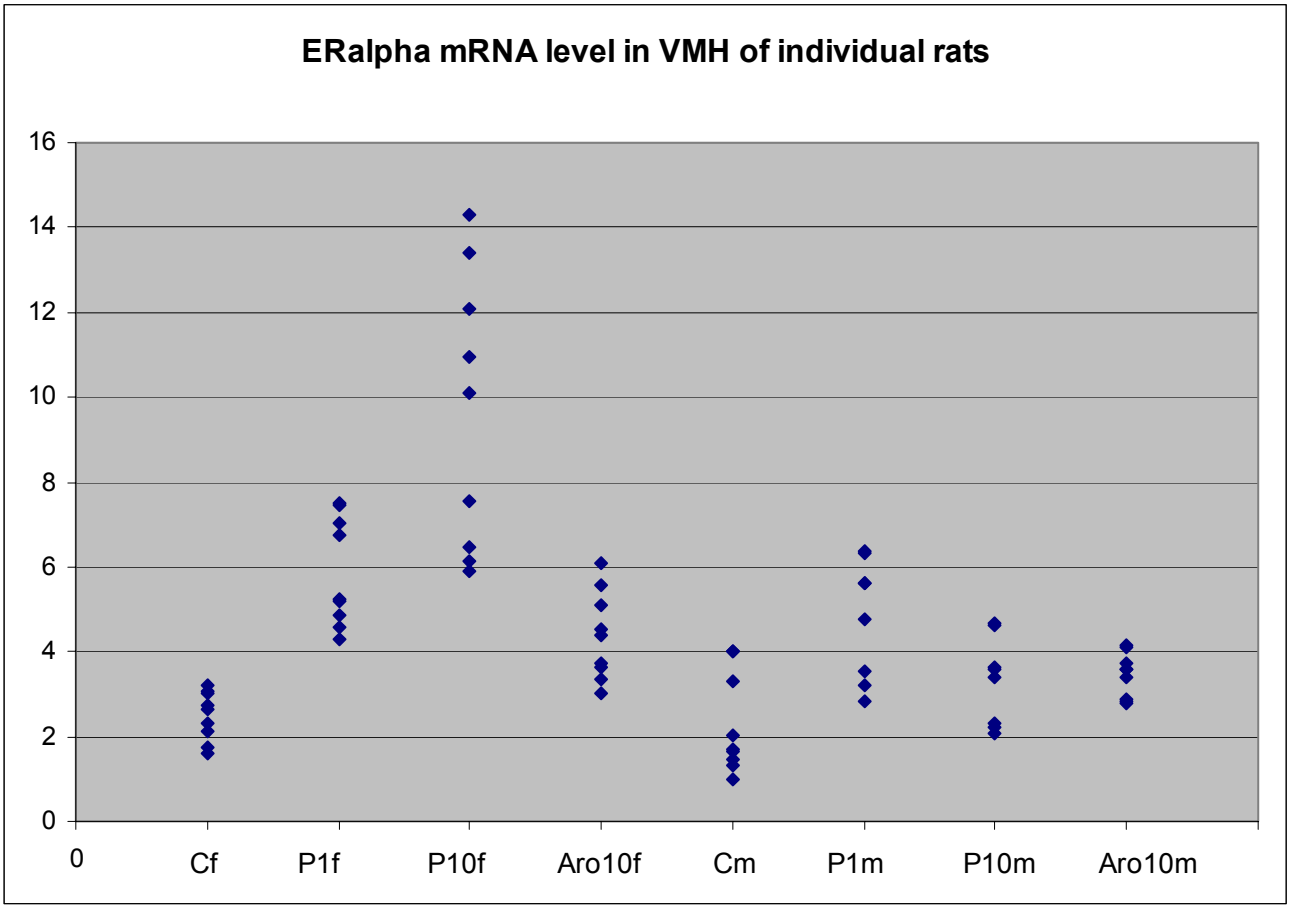


Fig. 43 Estrogen receptor alpha (ER alpha) mRNA level in the ventromedial hypothalamus (VMH) of individual animals. PR mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Estrogen receptor beta mRNA in VMH

Shughruhe et al. (1997) have shown, that the estrogen receptor beta is not much expressed in the VMH. However, ER beta mRNA was detectable. Although the level was already very low in the controls, there was a significant decrease in the two PBDE99 groups in both sexes, whereas the PCB-mixture (Aroclor 1254) did not have a significant effect.

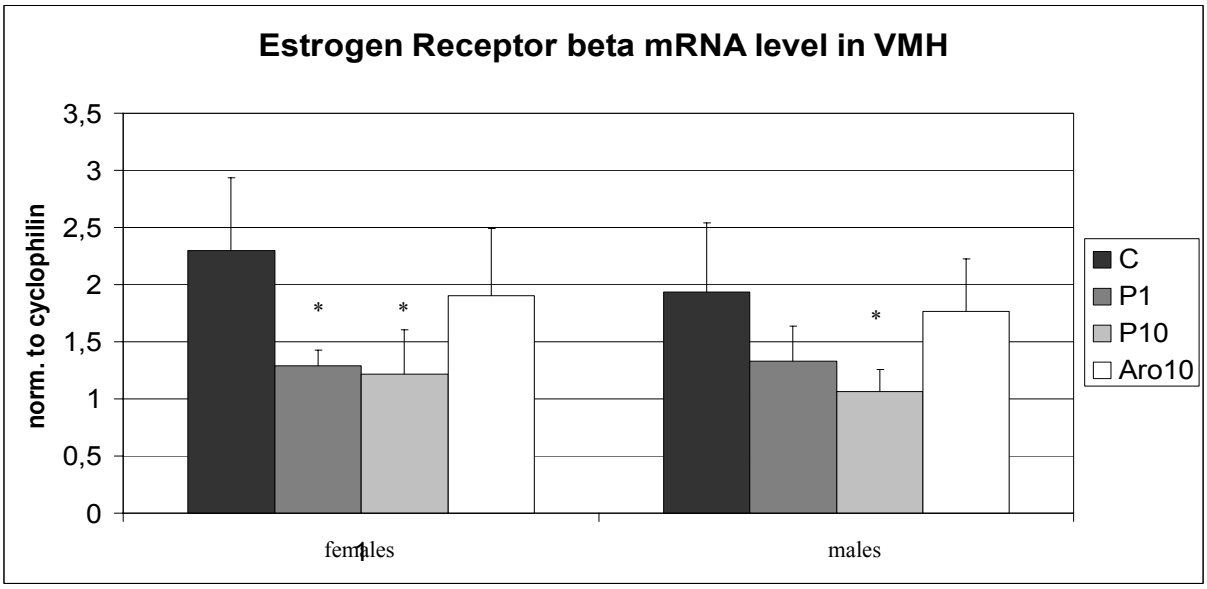


Fig. 44 Estrogen receptor beta (ER beta) mRNA level in the ventromedial hypothalamus (VMH) of adult male and female rat offspring. Mean ± S.D. (n=9) mRNA level of ER beta, normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of mRNA level compared to control are indicated by * ($p<0.05$)

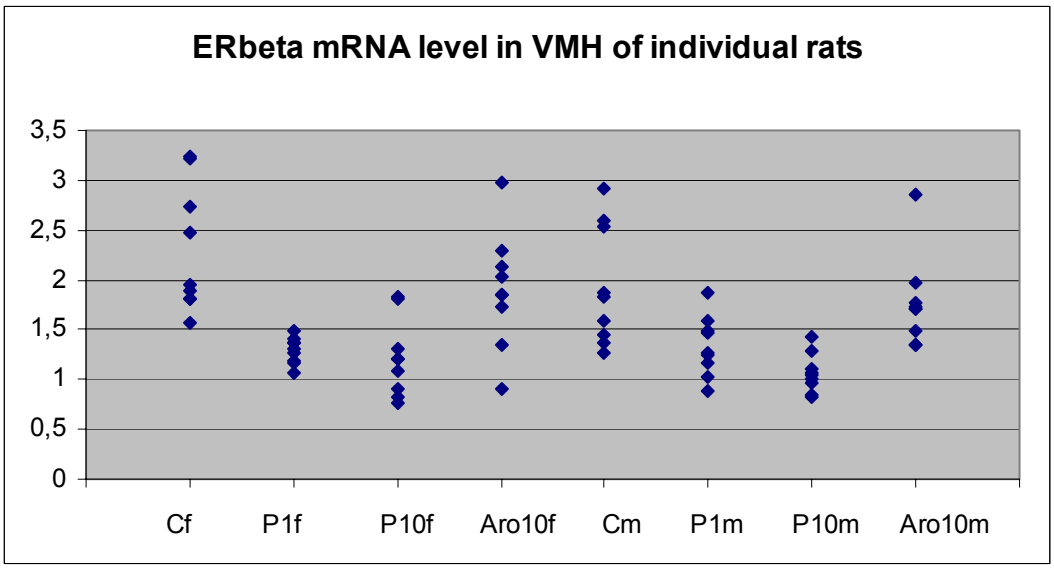


Fig. 45 Estrogen receptor beta (ER beta) mRNA level in the ventromedial hypothalamus (VMH) of individual animals. ER beta mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Preproenkephalin mRNA in VMH

The preproenkephalin mRNA level shows no difference between the sexes. This remains throughout all treatments. mRNA levels increase significantly in the PBDE groups and decrease in the PCB group.

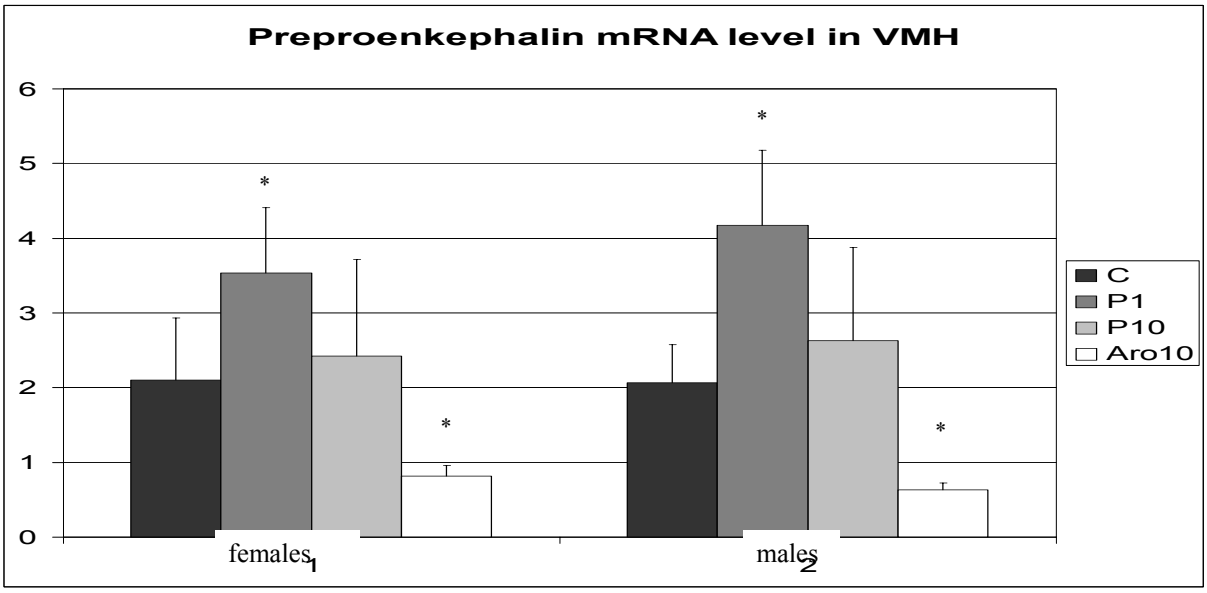


Fig. 46 Preproenkephalin (PPE) mRNA expression in the ventromedial hypothalamus (VMH) of adult male and female F1 rat offspring. Mean ± S.D. (n=9) mRNA level of preproenkephalin normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of mRNA level compared to controls are indicated by * ($p<0.05$).

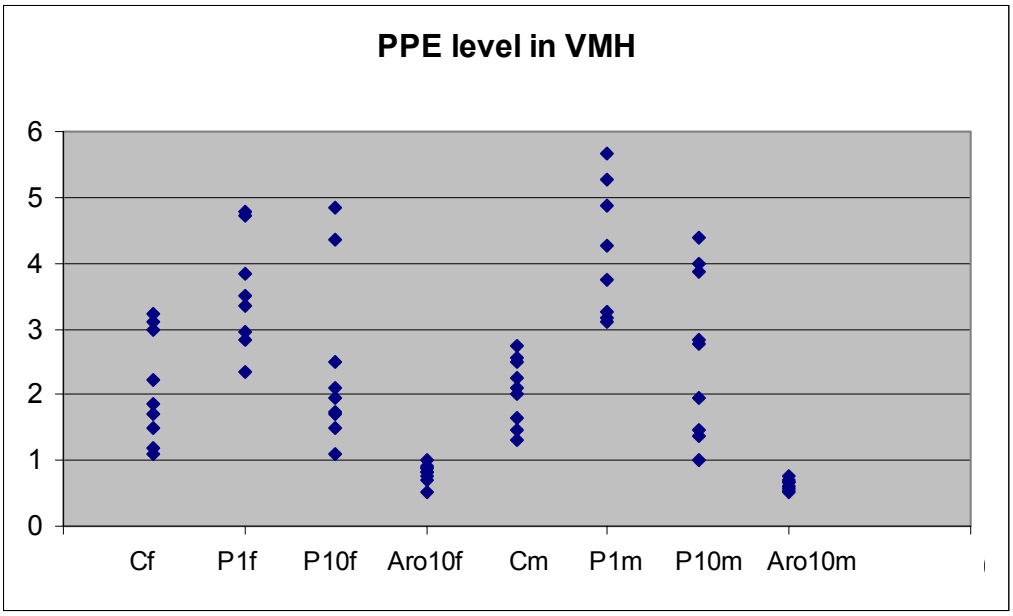


Fig. 47 PPE mRNA level in the ventromedial hypothalamus (VMH) of individual animals. PPE mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Medial preoptic area: baseline mRNA expression in adult male and female F1 offspring

This chapter describes the results of the molecular biological analysis in the medial preoptic area (MPO) for the adult F1 generation under steady state conditions (baseline).

Progesterone receptor mRNA in MPO

The mRNA level of the progesterone receptor in the MPO shows no significant difference between males and females in the control group, and the treatment with either PBDE or PCB does not change this significantly.

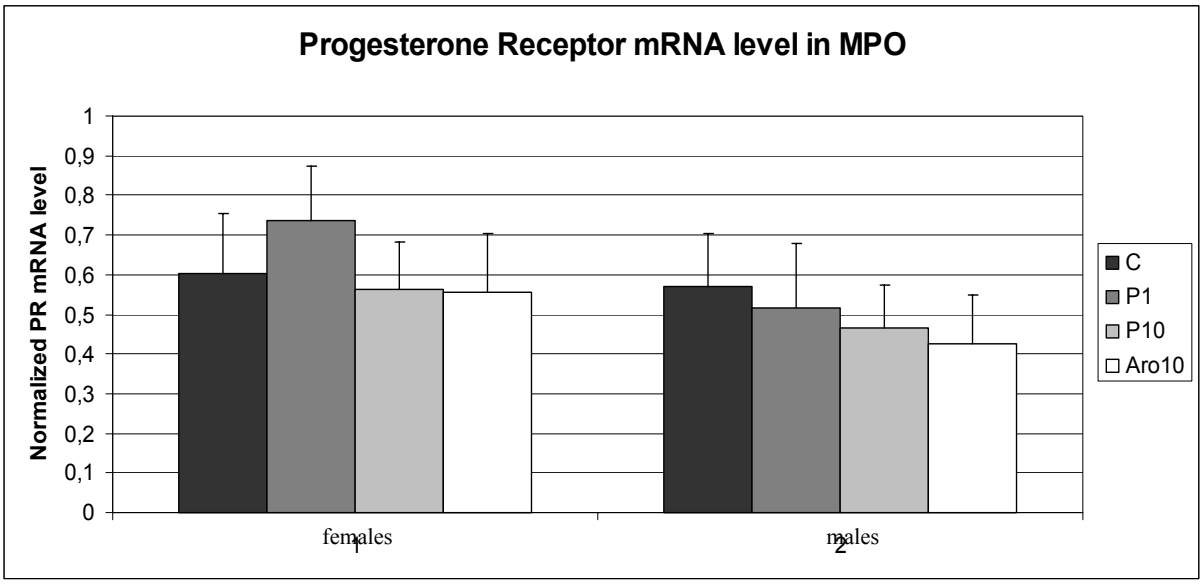


Fig. 48 Progesterone receptor (PR) mRNA expression in the medial preoptic area (MPO) of adult male and female F1 rat offspring. Mean ± S.D. (n=9) mRNA level of the progesterone receptor normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of the mRNA expression compared to controls are indicated by * ($p<0.05$).

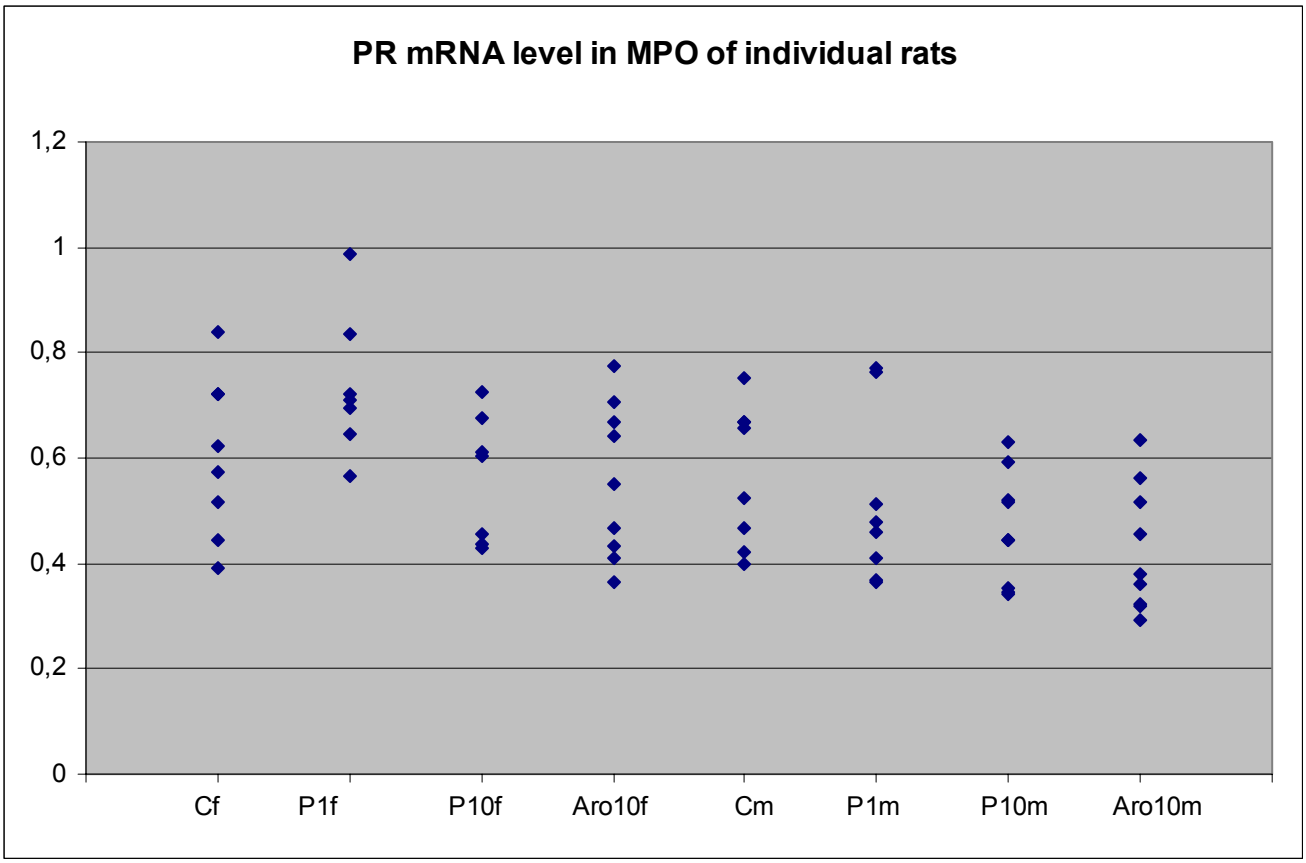


Fig. 49 PR mRNA level in the MPO of individual animals. PR mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Estrogen receptor alpha mRNA in MPO

In the control animals there is no sex difference detectable in the mRNA level of estrogen receptor alpha in the MPO. This remains throughout all treatment groups. The P10 group exhibited a dramatic and significant increase of ER alpha mRNA. P1 values tended to decrease, but this change was not significant. The PCB mixture was ineffective. Males and females of this treatment group showed different amounts of this gene's mRNA, however, this difference is not significant due to the high standard deviation.

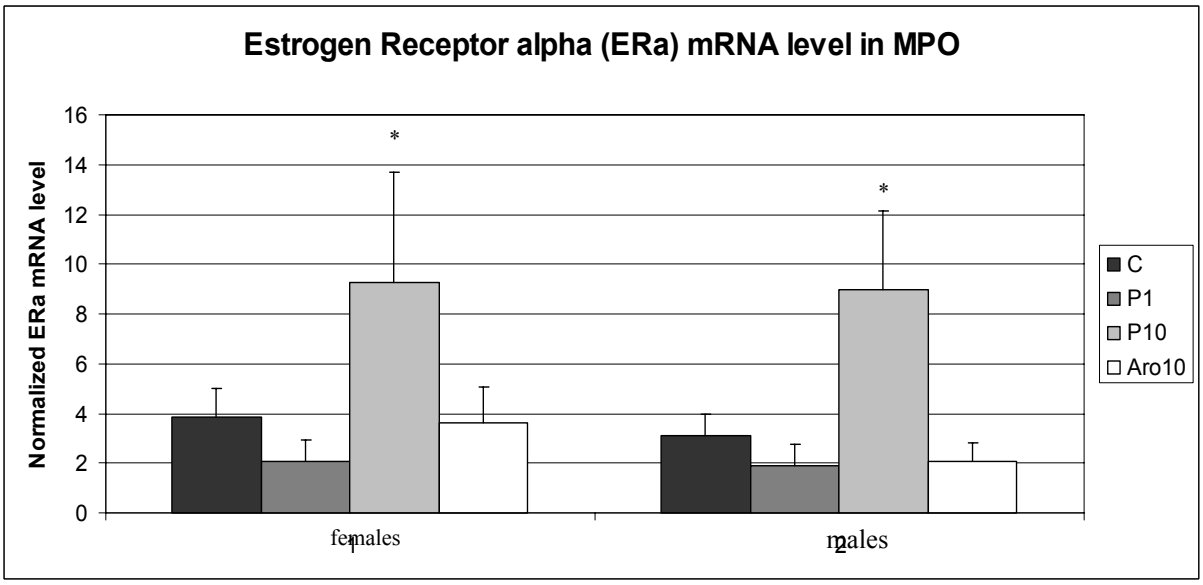


Fig. 50 Estrogen receptor alpha (ER alpha) mRNA expression in medial preoptic area (MPO) of male and female of adult F1 rat offspring. Mean ± S.D. (n=9) mRNA level of the estrogen receptor alpha normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of the mRNA expression compared to controls are indicated by * ($p<0.05$).

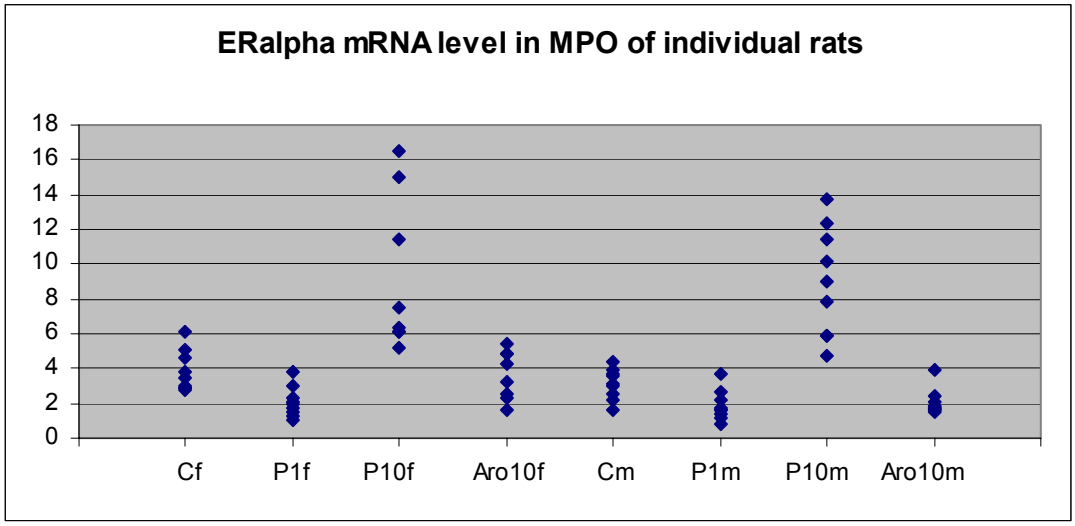


Fig. 51 ERalpha mRNA level in the MPO of individual animals. ERa mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Estrogen receptor beta mRNA in MPO

PBDE treatment did not significantly change the mRNA level of estrogen receptor beta in the MPO in contrast to PCB treatment, which increased ER beta mRNA level considerably in females.

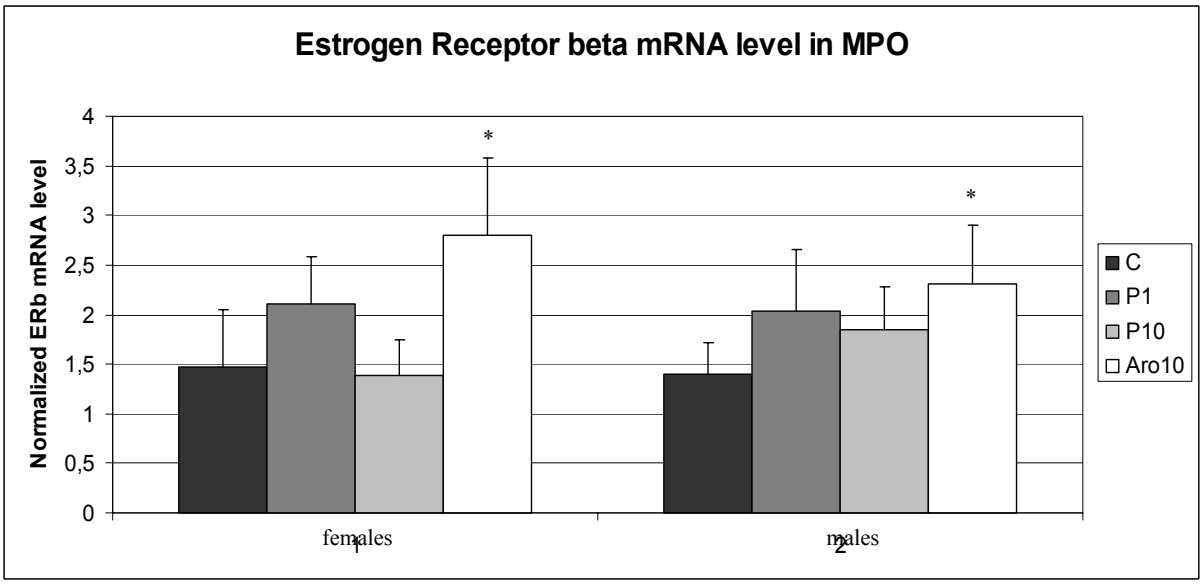


Fig. 52 Estrogen receptor beta (ER beta) mRNA expression in medial preoptic area (MPO) of male and female of adult F1 rat offspring. Mean ± S.D. (n=9) mRNA level of the estrogen receptor beta normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of the mRNA expression compared to controls are indicated by * ($p<0.05$).

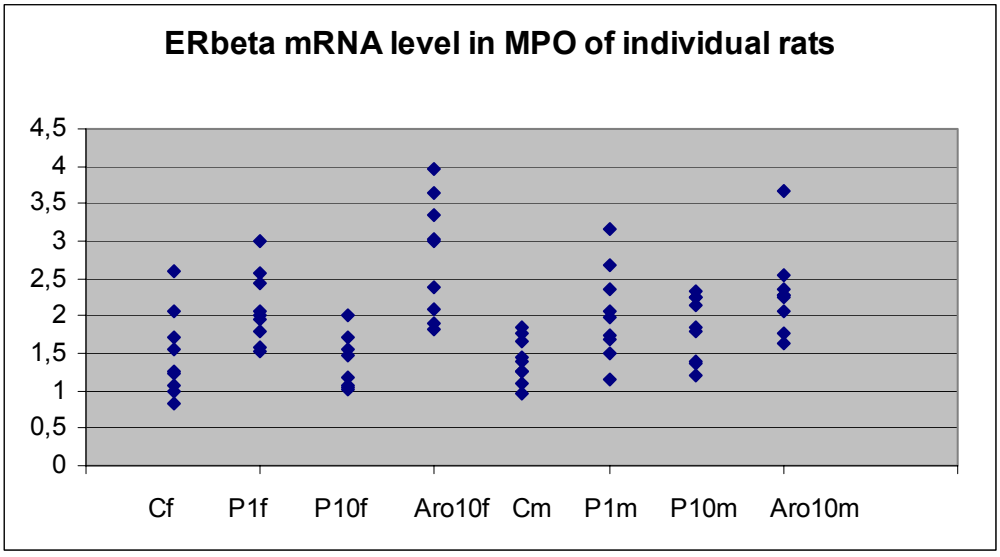


Fig. 53 ERbeta mRNA level in the MPO of individual animals. ERb mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

Preproenkephalin mRNA in MPO

In the MPO of females as well as males the mRNA level of preproenkephalin decreased after both PBDE99 and PCB treatment.

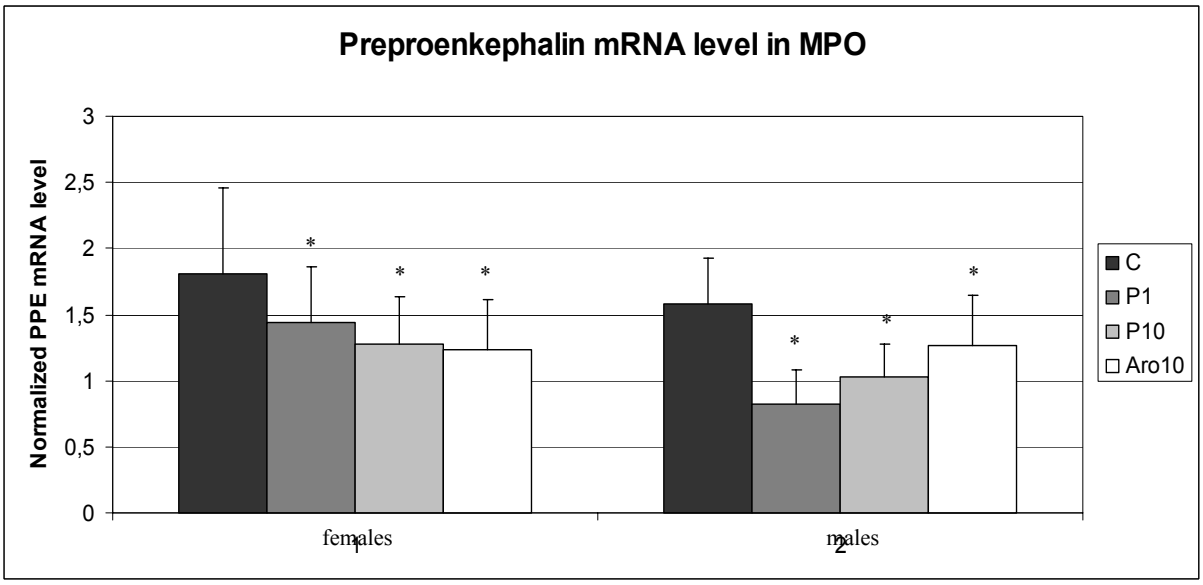


Fig. 54 Preproenkephalin (PPE) mRNA expression in medial preoptic area (MPO) of male and female of adult F1 rat offspring. Mean ± S.D. (n=9) mRNA level of the PPE normalized to cyclophilin (Cyc) mRNA. Statistically significant differences of the mRNA expression compared to controls are indicated by * ($p<0.05$).

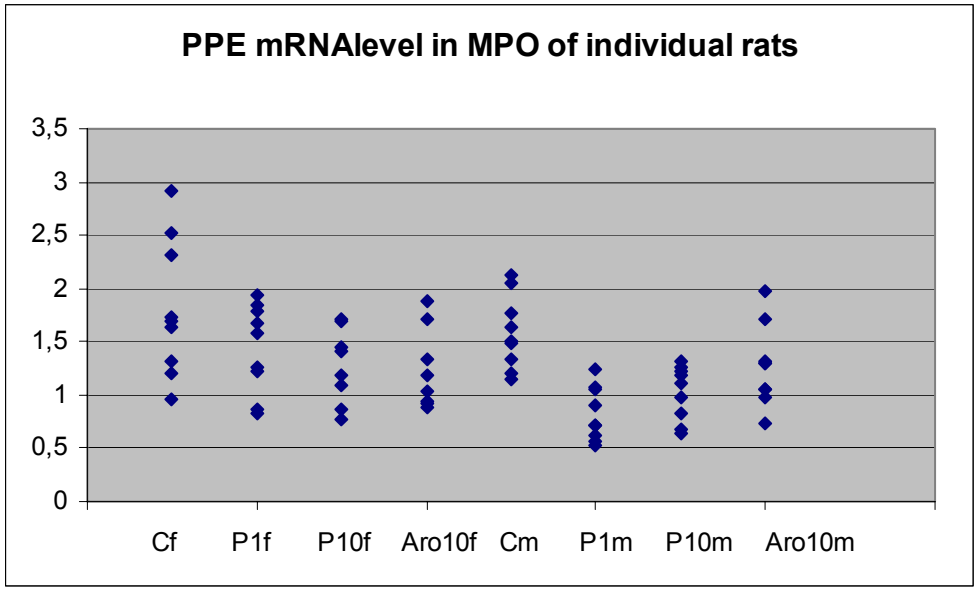


Fig. 55 PPE mRNA level in the MPO of individual animals. PPE mRNA level is normalized to cyclophilin (Cyc). (n= 9 per group).

E. Estrogen Challenge Experiments

mRNA expression in VMH of adult gonadectomized male and female F1 rat offspring after a single dose of estradiol

Possible changes in the sensitivity of genes for estrogen is another interesting endpoint for assessing the impact of endocrine disrupting chemicals during development. To further investigate this question, adult F1 offspring of both sexes were gonadectomized at 10 weeks of age in order to remove the internal production of sex steroids thus achieving a low, constant level. Two weeks later the animals were challenged with a single dose of estradiol, given by subcutaneous injection. mRNA levels of Progesterone Receptor (PR) and Preproenkephalin (PPE) were determined in VMH or MPO 6 hrs after the estradiol injection.

PR mRNA in the ventromedial hypothalamus (VMH) after acute estrogen treatment

Untreated control males exhibited a significant increase of PR mRNA level after treatment with estradiol, as compared to vehicle-injected controls. The same was true for animals prenatally treated with PBDE99 (10mg/kg/d). In contrast, the PBDE99 low dose group (1mg/kg/d) and the Aroclor1254 groups showed no induction of PR mRNA by estradiol. Differences between mRNA levels of vehicle-injected animals were observed for females treated with the PCB-mixture and for the males of both PBDE99 treated groups.

In the VMH of females estradiol also caused a significant induction of PR mRNA. It appeared to be greater than in males. The sensitivity of PR to estradiol was dose-dependently reduced by PBDE 99 with a significant reduction in the PBDE high dose group (P10), and in Aroclor-exposed offspring.

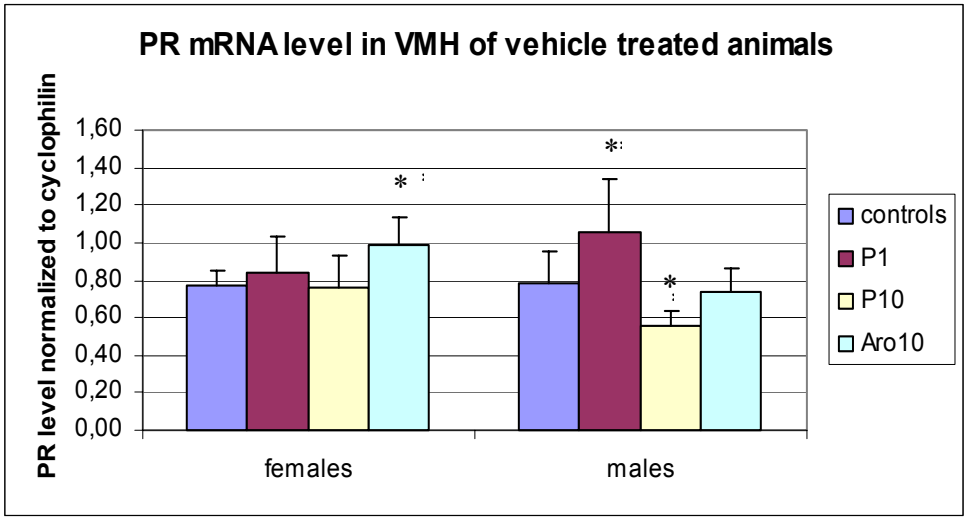


Fig. 56 Normalized mRNA level of progesterone receptor (PR) in the ventromedial hypothalamus (VMH) of adult gonadectomized F1 offspring injected with vehicle (n = 9) Mean values ±S.D. Significant differences between PBDE- or PCB-exposed groups and controls are indicated by * ($p<0.05$)

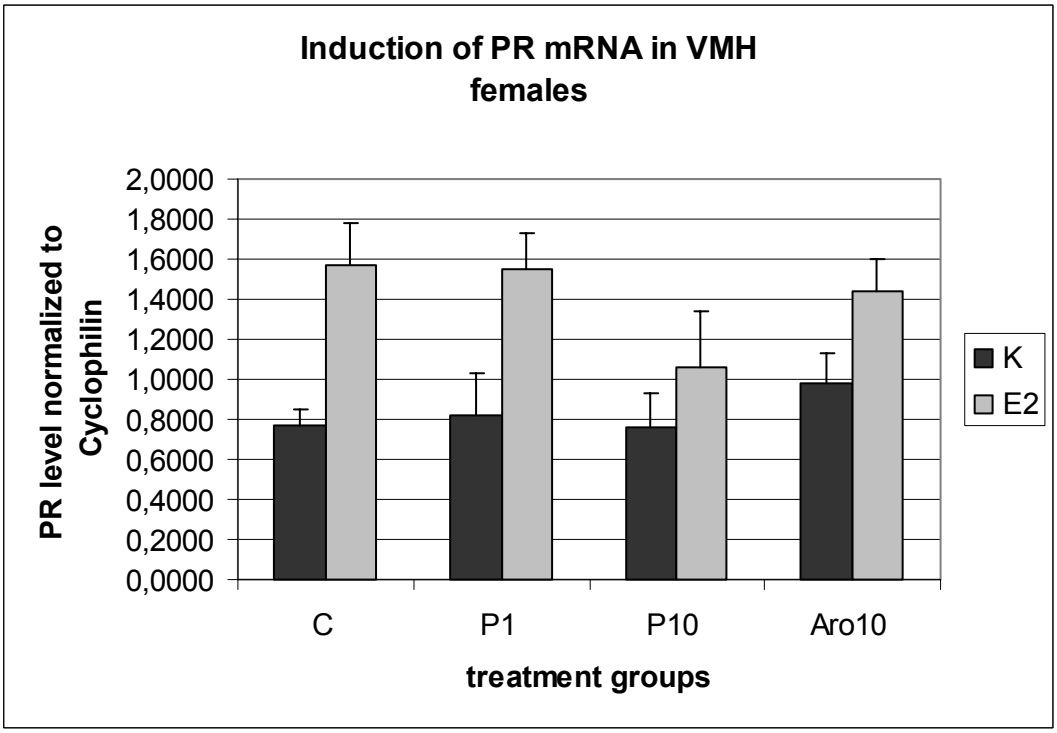


Fig. 57a Normalized mRNA level of progesterone receptor (PR) in the ventromedial hypothalamus (VMH) of gonadectomized females (n = 9) 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight) or vehicle (K). Mean values ±S.D. Significant differences between E2 and vehicle treated animals are indicated by * ($p<0.05$)

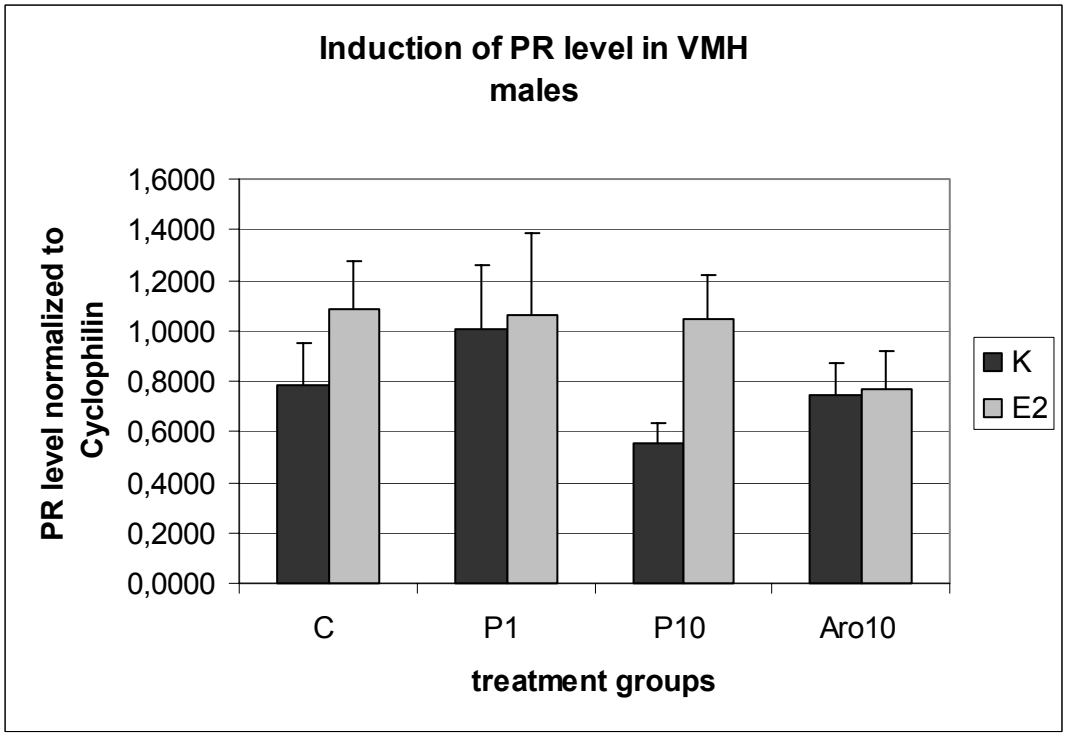


Fig. 57b Normalized mRNA level of progesterone receptor (PR) in the ventromedial hypothalamus (VMH) of gonadectomized males (n = 9) 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight) or vehicle (K). Mean values ±S.D. Significant differences between E2 and vehicle treated animals are indicated by * ($p<0.05$)

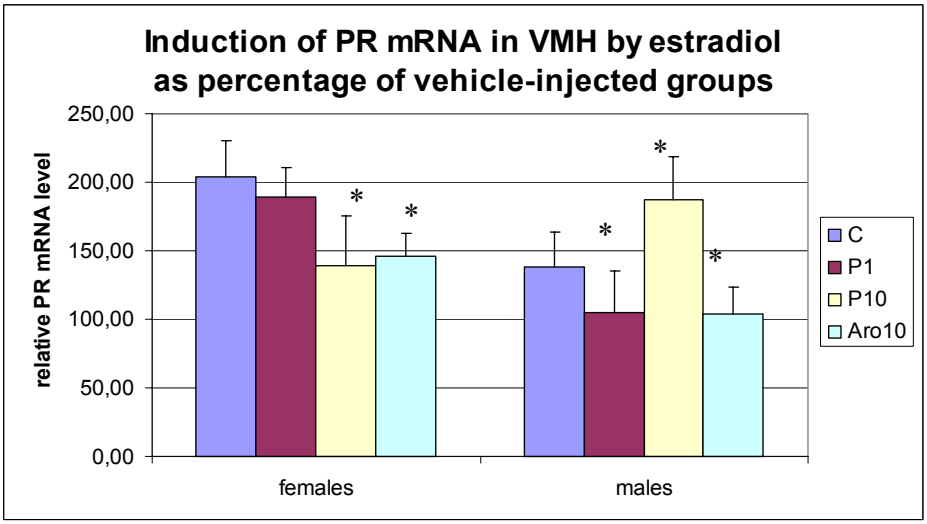


Fig. 57c Progesterone receptor (PR) mRNA levels in the ventromedial hypothalamus (VMH) of adult gonadectomized F1 offspring (n = 9) 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight) expressed as percentage of the corresponding vehicle-injected group of each chronic treatment group. Mean values ±S.D. Significant differences in mRNA induction by estradiol between PBDE- or PCB-exposed groups and untreated controls are indicated by * ($p<0.05$)

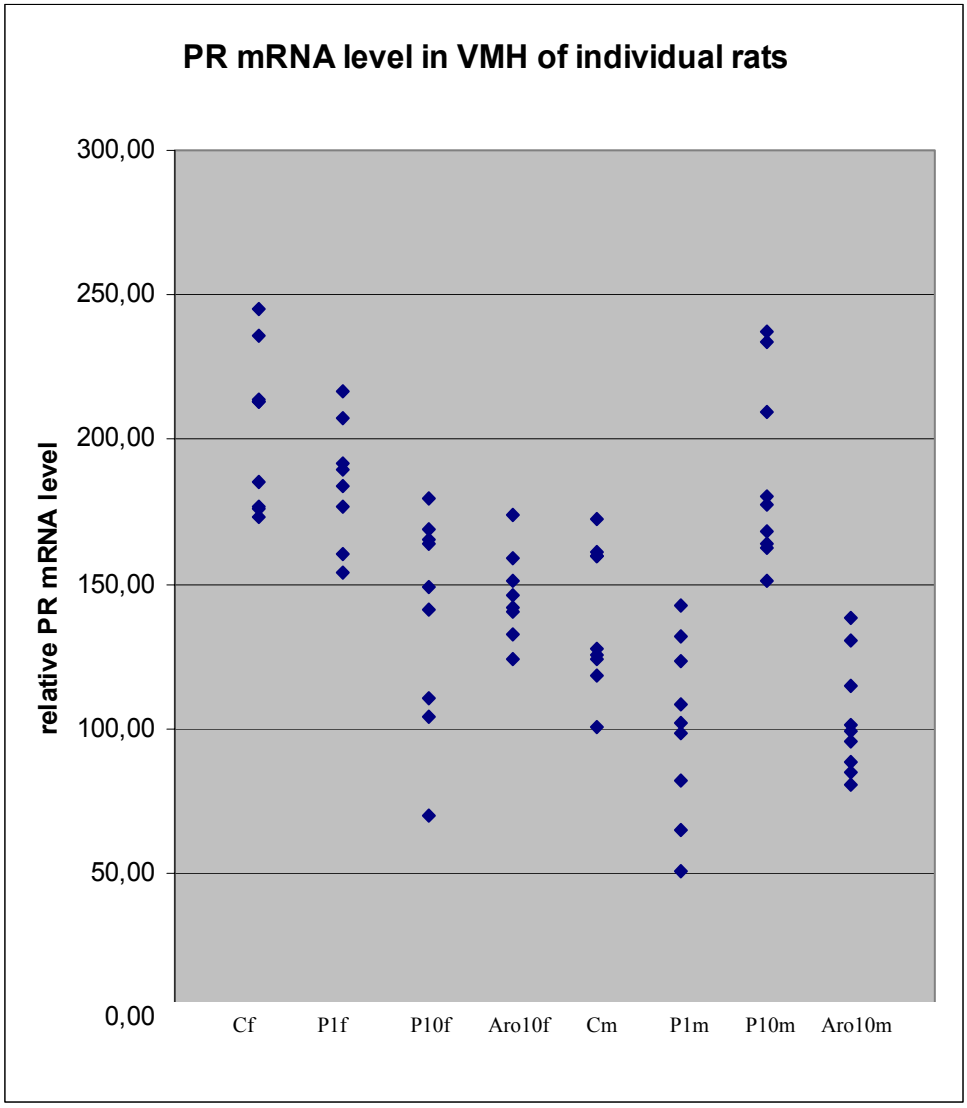


Fig. 58 Relative PR mRNA level in the VMH of individual gonadectomized rats 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight). The PR mRNA level is normalized to cyclophilin (Cyc). (n = 9 per group).

PPE mRNA in VMH after acute estrogen treatment

The mRNA level of PPE in the VMH of male offspring of untreated controls was not significantly affected by acute estradiol injection. The same was true for the PCB treated group (Aro10). Both PBDE99 groups showed a reduction of PPE mRNA by estradiol to about 50% of the control level. However, the PPE mRNA levels of vehicle controls was about 5fold higher in the 1mg/kg/d PBDE99 group as compared to the untreated control group, but similar to that of untreated controls in the 10mg/kg/d PBDE99 group. In the female VMH estradiol reduced the PPE mRNA level only in the 10mg/kg/d PBDE99 group.

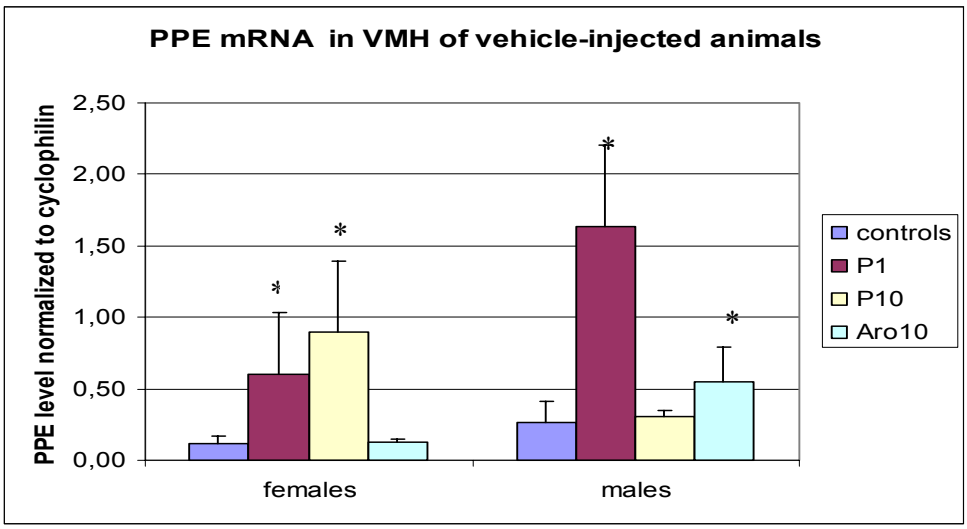


Fig. 59 Normalized mRNA level of preproenkephalin (PPE) in the ventromedial hypothalamus (VMH) of adult gonadectomized F1 offspring injected with vehicle (n = 9) Mean values ±S.D. Significant differences between PBDE- or PCB-exposed groups and controls are indicated by * ($p<0.05$)

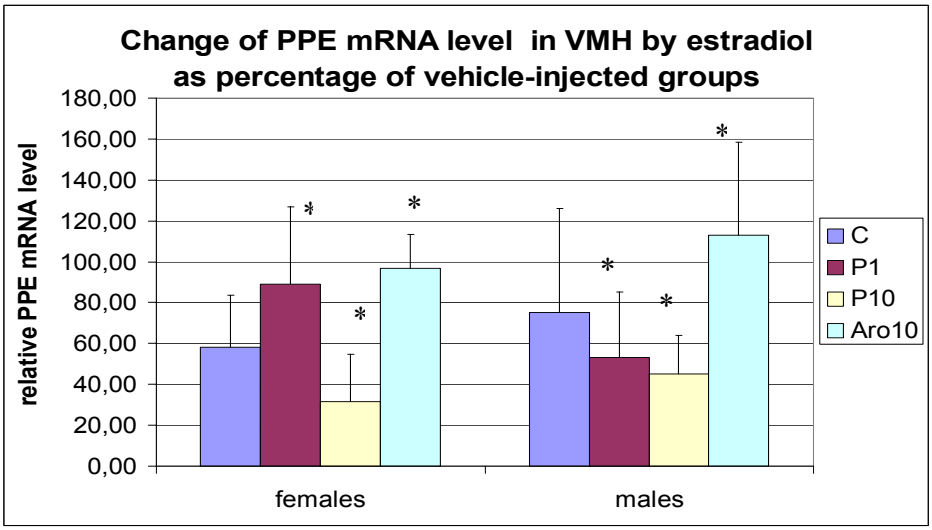


Fig. 60 Preproenkephalin (PPE) mRNA levels in the ventromedial hypothalamus (VMH) of adult gonadectomized F1 offspring (n = 9) 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight) expressed as percentage of the corresponding vehicle-injected group of each chronic treatment group. Mean values ±S.D. Significant differences in mRNA induction by estradiol between PBDE- or PCB-exposed groups and untreated controls are indicated by * ($p<0.05$)

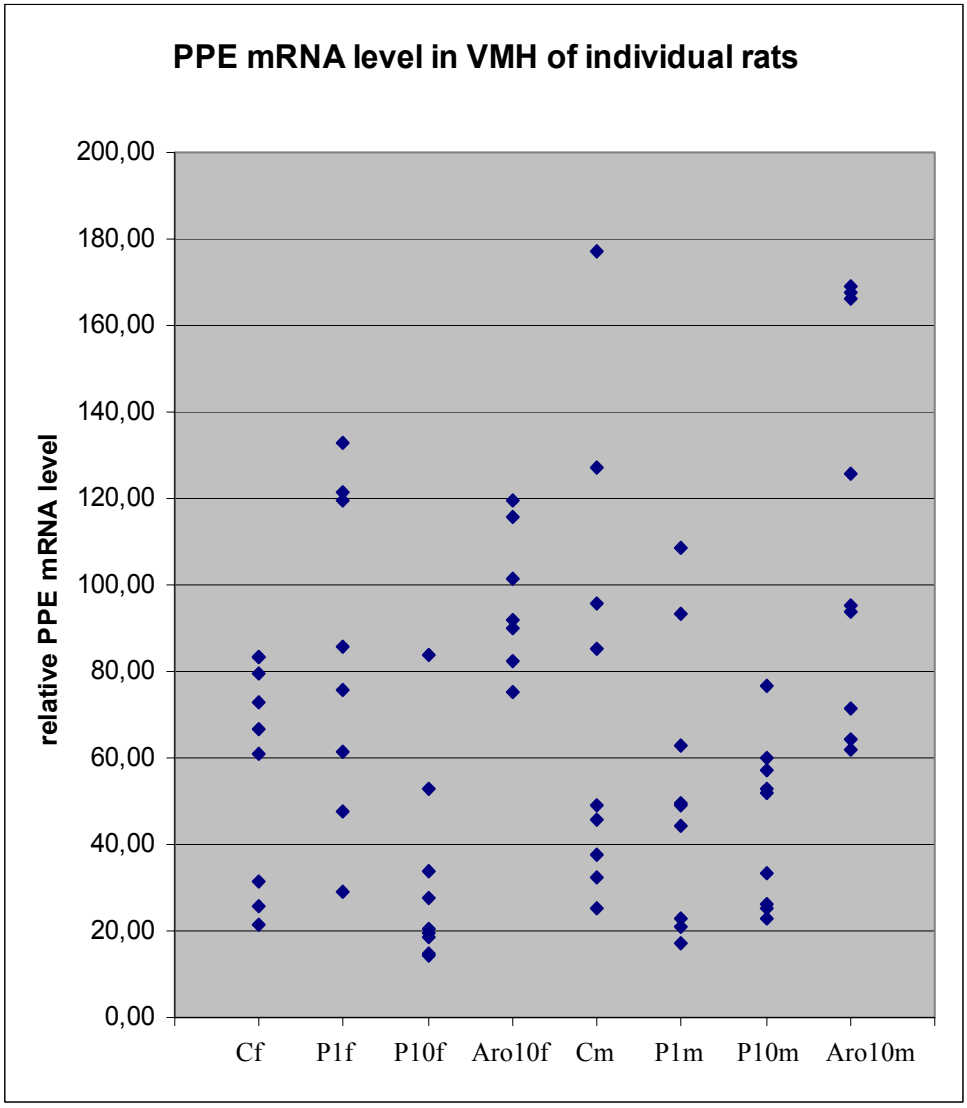


Fig. 61 Relative PPE mRNA level in the VMH of individual gonadectomized rats 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight). The PPE mRNA level is normalized to cyclophilin (Cyc). (n = 9 per group).

mRNA expression in MPO of adult gonadectomized male and female rat offspring after acute estrogen challenge

PR mRNA level in MPO after acute estrogen treatment

Acute estradiol injection gives rise to a small but significant increase of progesterone receptor mRNA level in untreated male controls when compared to the corresponding vehicle group. After treatment with PBDE99 this effect is abolished with both groups (P1 and P10). Both PBDE99 treatments resulted in elevated PR mRNA levels in the vehicle control groups. In the PCB-treated group (Aro10) the effect of estradiol seems to be reversed, since the estradiol treated animals now show a significantly lower level of PR mRNA in the MPO than the vehicle treated ones.

The original 50% induction of PR mRNA by estradiol in the MPO of the control group was abolished in all treatment groups (PBDE and PCB). Only the control level seems to be different in these two groups: PBDE treatment did not affect the control level (vehicle controls), where PCB increased control levels.

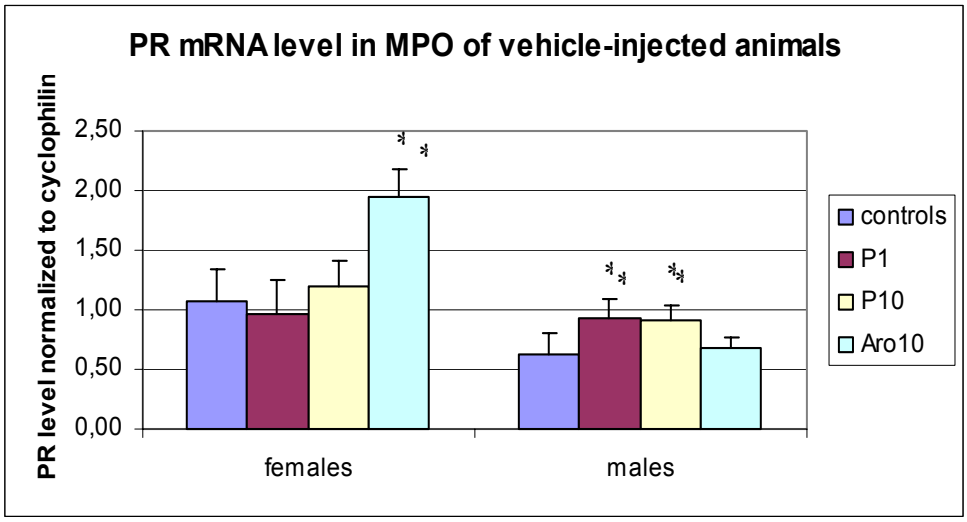


Fig. 62 Normalized mRNA level of progesterone receptor (PR) in the medial preoptic area (MPO) of adult gonadectomized F1 offspring injected with vehicle (n = 9) Mean values ±S.D. Significant differences between PBDE- or PCB-exposed groups and controls are indicated by * ($p<0.05$)

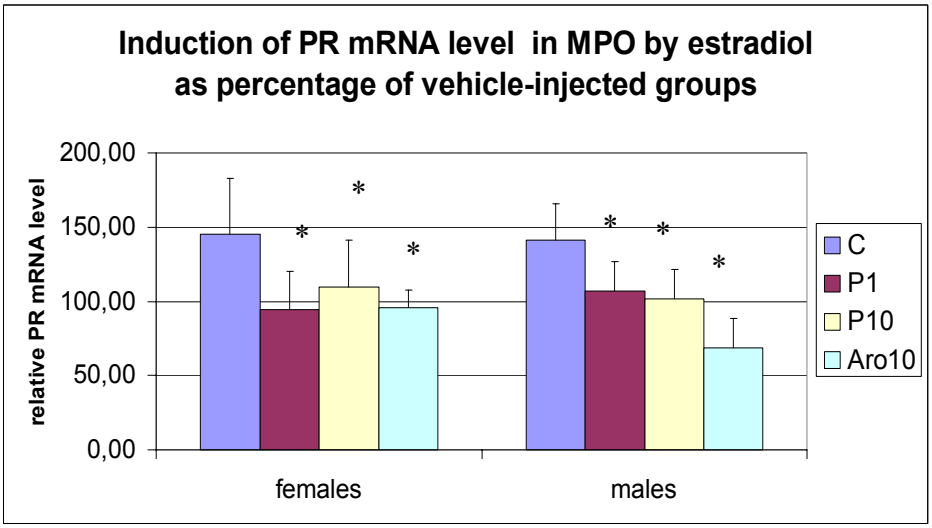


Fig. 63 Progesterone receptor (PR) mRNA levels in the medial preoptic area (MPO) of adult gonadectomized F1 offspring (n = 9) 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight) expressed as percentage of the corresponding vehicle-injected group of each chronic treatment group. Mean values ±S.D. Significant differences in mRNA induction by estradiol between PBDE- or PCB-exposed groups and untreated controls are indicated by * ($p<0.05$)

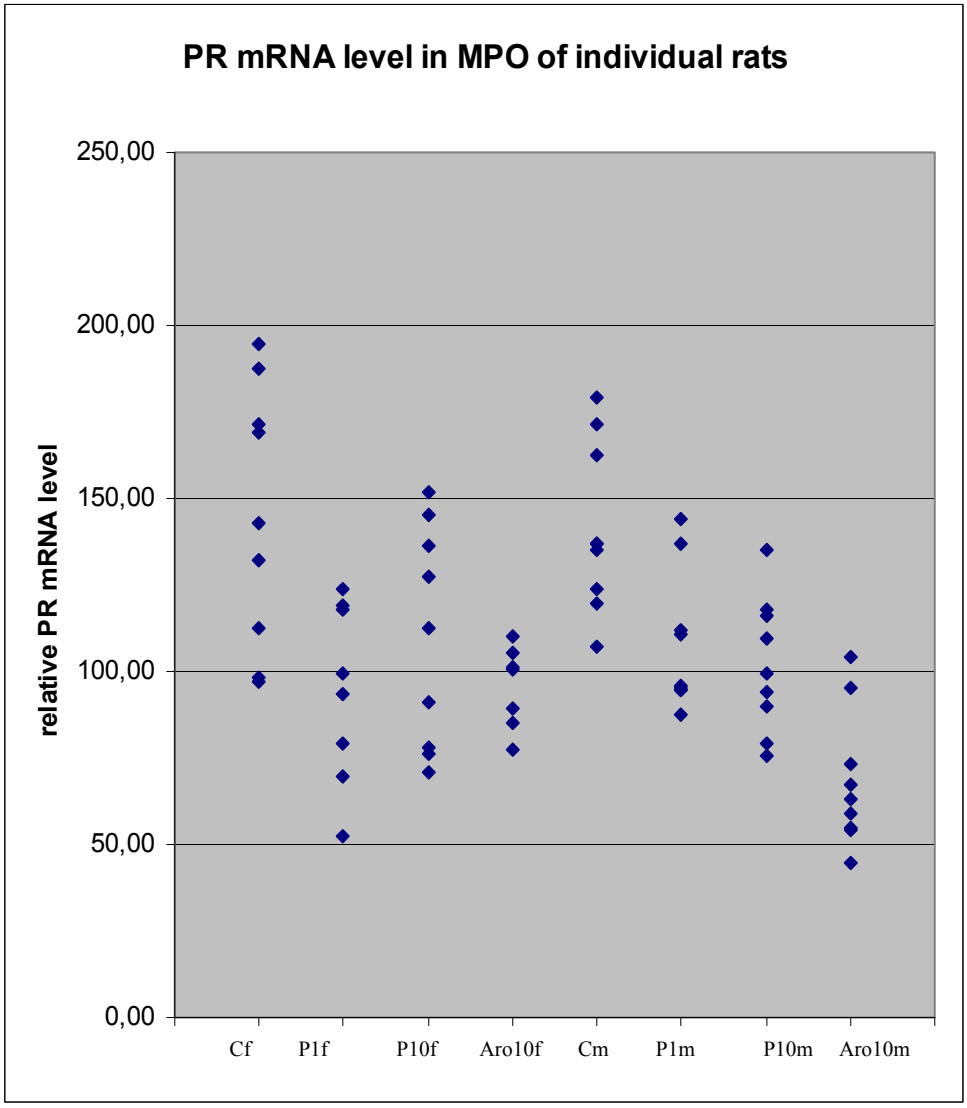


Fig. 64 Relative PR mRNA level in the MPO of individual gonadectomized rats 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight). The PR mRNA level is normalized to cyclophilin (Cyc). (n = 9 per group).

PPE mRNA in MPO after acute estrogen treatment

Estradiol treatment of gonadectomized F1 males leads to a significant decrease of PPE mRNA level in the MPO of the control group. This effect is not altered after treatment with a low dose of PBDE99 (P1), but the higher dose (10mg/kg) abolished the repressive effect of estradiol. PCBs similarly abolished the repression of PPE mRNA by estradiol. PPE mRNA levels in vehicle-injected males exposed to PBDE99 or PCB were lower than those of vehicle injected controls.

Estradiol treatment tended to reduce PPE mRNA levels in the MPO of female controls and 1mg/kg PBDE99-exposed animals, but the effect was not significant. In the P10 and Aro10 groups, estradiol was ineffective. PPE mRNA levels of vehicle-injected groups did not differ significantly from each other.

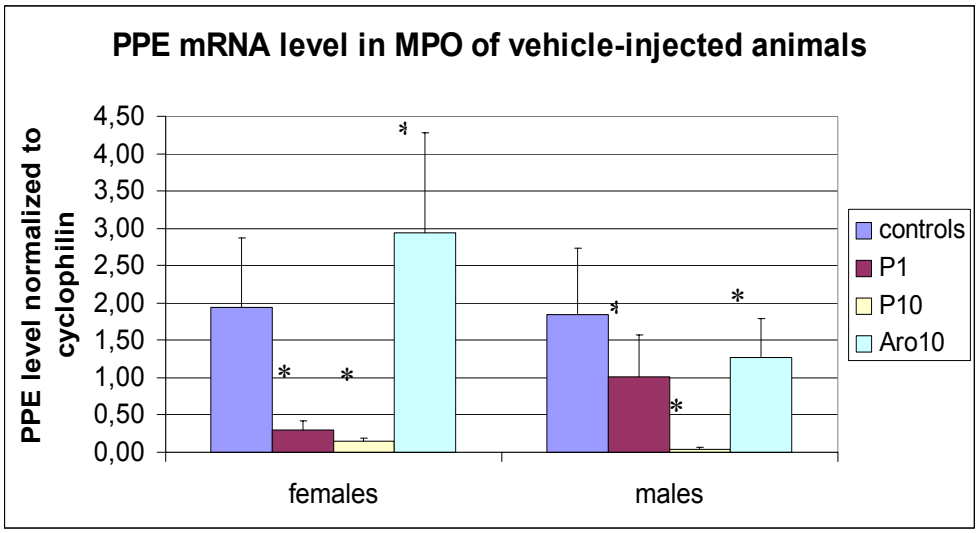


Fig. 65 Normalized mRNA level of preproenkephalin (PPE) in the medial preoptic area (MPO) of adult gonadectomized F1 offspring injected with vehicle (n = 9) Mean values ±S.D. Significant differences between PBDE- or PCB-exposed groups and controls are indicated by * ($p<0.05$)

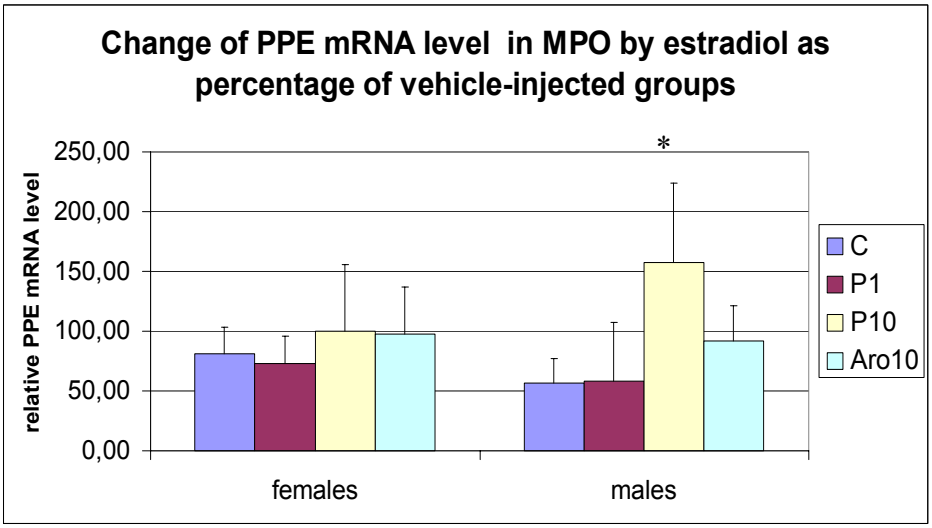


Fig. 66 Preproenkephalin (PPE) mRNA levels in the medial preoptic area (MPO) of one single dose of estradiol (E2) (10 µg/kg body weight) expressed as percentage of the corresponding vehicle-injected group of each chronic treatment group. Mean values ±S.D. Significant differences in mRNA induction by estradiol between PBDE- or PCB-exposed groups and untreated controls are indicated by * ($p<0.05$)

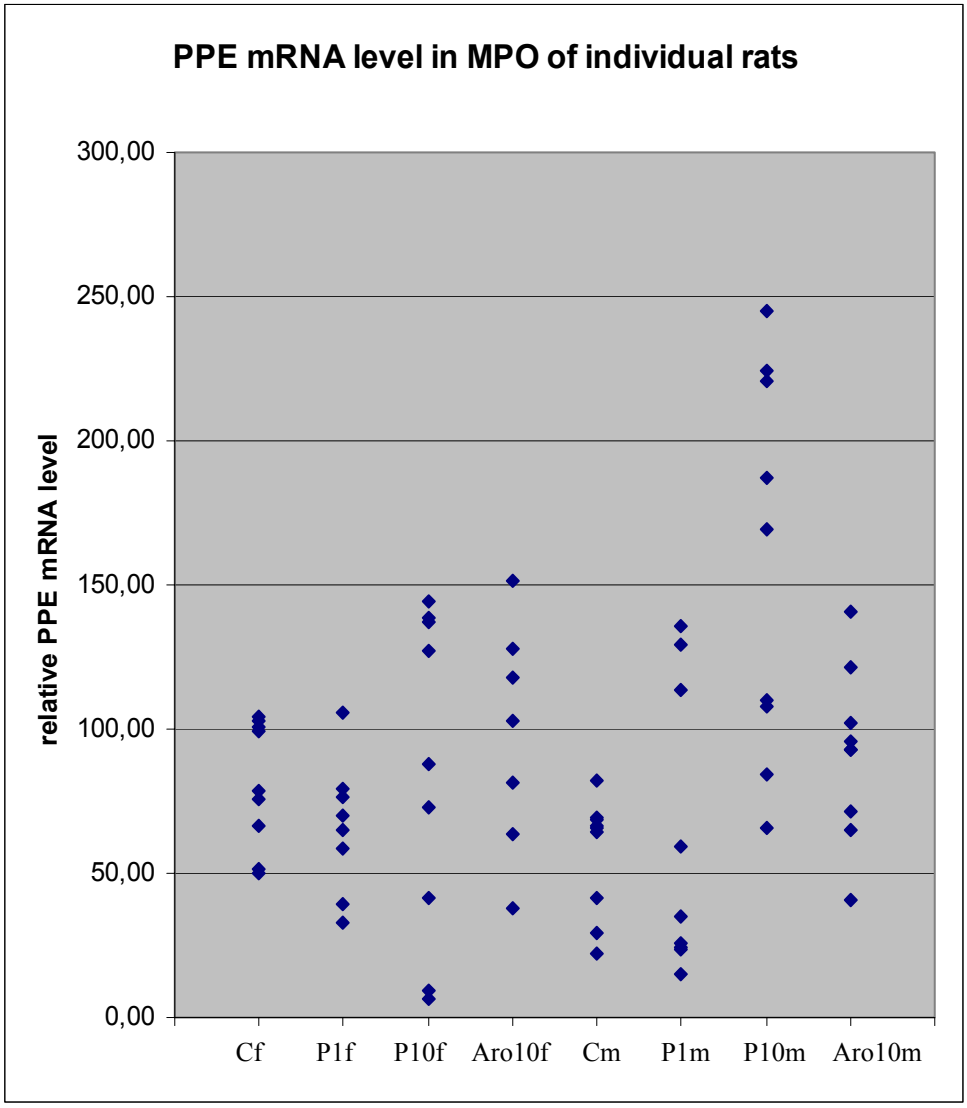


Fig. 67 Relative PPE mRNA level in the MPO of individual gonadectomized rats 6 hours after subcutaneous injection of one single dose of estradiol (E2) (10 µg/kg body weight). The PPE mRNA level is normalized to cyclophilin (Cyc). (n = 9 per group).

Synopsis of molecular results of estrogen target gene mRNA expression under steady state conditions (baseline)

The final two tables are meant to give an overview of the results of the mRNA levels in estrogen target genes in adult rat offspring under steady state conditions (table 8) and for the estrogene challenge experiment (table 9), respectively. Shown are significant increase (↑) or decrease (↓) of mRNA levels of the examined genes as compared to the respective control group.

It illustrates, that developmental exposure to either PBDEs or PCBs alter the regulation of mRNA levels in both brain regions of both sexes. The mode of action, however differs between PBDEs and PCBs.

sex	treatment group	MPO				VMH			
females	P1	-	-	-	-	↑	↓	↑	↓
	P10	↑	-	↓	-	↑	↓	-	↓
	Aro10	-	↑	↓	-	-	-	↓	↓
males	P1	-	-	↓	-	↑	-	↑	↑
	P10	↑	-	↓	-	-	↓	-	-
	Aro10	-	↑	-	-	-	-	↓	-
genes		ER alpha	ER beta	PPE	PR	ER alpha	ER beta	PPE	PR

Tab. 8 Synopsis of mRNA levels in adult offspring after prenatal treatment with PBDE99 or a PCB-mixture (Aroclor1254): ↑ = increase, ↓ = decrease, - = no change as compared to respective controls

		PR				PPE			
		C	P1	P10	Aro10	C	P1	P10	Aro10
females	MPO	↑	↓	↑	↓	↓	↓	-	↓
	VMH	↑	↑	↑	↑	↓	↓	↓	↓
males	MPO	↑	↑	↑	↓	↓	↓	↑	↓
	VMH	↑	↑	↑	↑	↓	↓	↓	↑

Tab. 9 Synopsis of progesterone receptor and preproenkephalin mRNA levels in response to acute estradiol injection in the different prenatal treated groups of adult gonadectomized F1 rat offspring.

DISCUSSION

In contrast to the rather well known effects of PCB on the developing nervous system very little is known about the effects of the structurally related PBDE. Despite the finding of very little effect in in-vitro systems (Meerts et al., 2001) the study at hand reveals some major effects of PBDE99 on brain development. It appears that the genetic program in sexually dimorphic brain regions of the rat is severely altered. This is a matter of concern as these regions are involved in the regulation of reproductive behavior (Gorski, 1980). As PBDE99 is one of the regularly detected brominated flame retardants in environmental samples, it has been selected for this study. The developmental toxicity of PBDE99 was evaluated with special regard to conventional as well as molecular toxicity parameters. As a comparative compound, the industrial PCB-mixture Aroclor1254 was added to this study. PCB are known to interfere with sexual brain differentiation (Hany et al., 1999).

The study revealed that PBDE99 can interfere with sexual brain differentiation processes at the molecular and functional level.

General toxicological parameters

Litter size and survival rate

With the exception of the high dose PCB group (30mg/kg Aroclor, Aro30) litter size and survival rate were not affected. This indicates that only this one dose showed unspecific toxic effects. Therefore the 30mg/kg Aroclor dosage group was eliminated in the subsequent molecular biology studies.

Body and organ weights

Body weights as well did not differ among the various treatment groups, again with the exception of the Aro30 group, with female bodyweights significantly lower than those of their age mates in controls and other treatment groups. Also, some females of the Aro30 group died at an age as early as around 1 month, and examinations post mortem pointed to the possibility that they may have suffered from waisting syndrome (data not shown).

As to organ weights the effect pattern differs between PCB treated animals and those treated with PBDE. The peripheral organs such as prostate and uterus are part of my colleague Raffaella Ceccatelli´s doctoral thesis (for further information see Ceccatelli, R. 2004).

With the exception of the Aro30 group these findings in summary revealed no unspecific toxicological effects at the levels chosen for this study.

Sexual development

Anogenital distance

Neither of the treatments showed any significant effect on the anogenital distance.

Onset of puberty

The onset of puberty, however, has been shifted in both sexes after treatment with the test compounds. Therefore it can be deduced that treatment has certain effects on sexual development.

Effects on gene expression

The present study revealed that pre- and postnatal exposure to either PBDE 99 or the PCB-mixture Aroclor 1254 results in long lasting changes in gene expression patterns of various estrogen-regulated genes (PR, ER alpha and beta and PPE) in sexually dimorphic brain regions of adult rat offspring. The data in females refer to diestrus day1 (metestrus).

Steady state conditions (baseline)

The first part of the examination of mRNA levels of estrogen-regulated genes in sexually dimorphic brain regions was done under steady state conditions (the so called baseline-experiment), which means that apart from the prenatal treatment with

pre- and postnatal exposure the animals underwent no further manipulation. Chemical analyses of adipose tissue in adult offspring showed that the animals were exposed also during postnatal ontogeny, as a result of the persistence of PBDE99 (unpublished observations) and PCB (Hany et al., 1999). A comparison with tissue levels for humans (She et al., 2002; Schecter et al., 2003; Sjödin et al., 2003) and marine mammals (She et al., 2002) showed the tissue levels of the lower dose PBDE99 group to come close to the upper concentration range of PBDEs in these reported natural samples.

Estrogen receptor alpha and beta mRNA expression in VMH and MPO of developmentally exposed adult offspring

The ER alpha mRNA levels in the VMH region of adult rat offspring in the control group were found to be sexually dimorphic with males (with the exception of two possible outliers) showing lower levels than females. This finding is in line with what has been reported by Shughrue and coworkers (1992). Sexual differences in estrogen receptor concentrations in specific brain regions are well known and have been reported first by Rainbow and coworkers (1982). They were able to detect higher binding levels of tritium-labeled estradiol in extracts of cytoplasm from micro-dissected female rat brain. The sexual difference may originate from the aromatization of testosterone during early postnatal life, when estradiol, derived from testosterone via aromatization by the enzyme aromatase, suppresses the expression of estrogen receptors. Aromatase mRNA levels in the brain are higher in early postnatal stages than in adulthood, and moreover, they are higher in certain forebrain regions of males as compared to their female counterparts (von Ziegler et al., 1991; Lauber and Lichtensteiger, 1994; Lauber et al., 1997).
Treatment with PBDE 99 as well as with the PCB-mixture led to an increase of ER alpha mRNA level in the VMH of both sexes. However, the relative sex difference persisted in all treatment groups despite the actual change in the absolute level of mRNA. In the MPO treatment with the high dose of PBDE99 affected ER alpha in a similar way as in VMH (up-regulation). The low dose of PBDE99 and Aroclor were ineffective. In contrast to Brown and co-workers (1988) we did not observe a significant sex difference in nuclear estrogen receptors.

Following acute exposure to estradiol Lauber and co-workers (1990) found a down-regulation of estrogen receptor sites in the ventromedial hypothalamic nucleus in female rats. The present data reflect the effect of long term exposure; whether they result from perinatal actions or from ongoing exposure, cannot be decided. PBDE99 was found to exhibit weak estrogenic activity (Meerts et al., 2001). Actions in the adult brain would thus be expected to decrease rather than increase ER alpha levels. Thus, the increase observed in exposed offspring might result from a developmental action.

ER beta mRNA levels were significantly reduced in VMH of both sexes by PBDE99, whereas the PCB mixture was ineffective. In MPO, in contrast the expression of this receptor was not affected by PBDE99, but up-regulated by Aroclor in females. This is one of the examples of qualitative differences in the effect patterns of the polybrominated diphenylether and polychlorinated biphenyl mixture. As in the case of ER alpha, it cannot be definitely decided whether the changes in gene expression resulted from early developmental or adult exposure.

The action of estrogen on transcription in neuronal development and function is mediated via estrogen receptors. Nuclear estrogen receptors function as ligand-modulated nuclear transcription factors, which regulate the expression of target genes, e. g. of sites in the genome encoding for neurotransmitters (Romano et al., 1989). ER alpha has been discovered in the 1960s in the brain of females. It took as long as to the middle of the 1990s until the second member of the steroid receptor superfamily named ER beta, has been discovered, first in the prostate (Kuiper et al., 1996) and later in brain tissue. The distribution patterns of these two estrogen receptors, ER alpha and ER beta, differ from brain region to brain region. ER alpha can be detected in distinct cell populations in the medial basal hypothalamus, including the VMH, as well as in the MPO of adult rats. ER beta is found in the MPO and other brain regions, levels in the VMH are low (Shughrue et al., 1997). It should be noted that particularly in central nervous system, estrogen receptors located in the cell membrane play a significant role (Minami et al., 1990; Abraham et al., 2003, 2004; Wade and Dorsa, 2003). This is true also for developmental processes (Beyer and Karolczak, 2000). Model systems based on binding to nuclear receptors or transcriptional effects would not reveal activities at membrane receptors. This might possibly explain why we observed drastic changes induced by PBDE99 even if this compound displays very little, if any, estrogenic activity in vitro (Meerts et al., 2000).

However, it is also conceivable that PBDE99 may have acted via mechanisms not involving sex steroid signaling.

Progesterone receptor mRNA expression in VMH and MPO of developmentally exposed adult offspring

Progesterone receptor (PR) mRNA levels in the VMH of controls showed a significant sex difference with a higher PR mRNA level in females than in males. This is in line with earlier reports that expression of PR is region- and sex-specific (Brown et al., 1987; Lauber et al. 1991). After treatment with either PBDE 99 or Aroclor1254, this sexual dimorphism disappeared. The effect was most clear-cut in the low dose PBDE group (P1), where the females´ level decreased and at the same time the level in males increased, yielding almost the same levels in both sexes. Female sexual behavior in rats depends directly on PR expression in VMH, as demonstrated by Ogawa and coworkers (1994), who observed an almost complete loss of the lordosis reaction of the female in response to mounting by a male, after local injection of antisense oligonucleotides against PR mRNA. The significant alteration in PR expression may be involved in the repression of female sex behavior observed in PBDE99-exposed offspring.
In MPO, PR mRNA level showed no significant sex-differences in controls and prenatal treatment with PBDE 99 as well as with PCB had no effect on the PR mRNA expression in the MPO. This again illustrates the regional specificity of xenobiotics-induced alterations in gene expression at brain level.

Preproenkephalin mRNA expression in VMH and MPO of developmentally exposed adult offspring

PPE is the precursor peptide of met-enkephalin and is expressed in the central nervous system at early developmental stages (Yamano et al., 1984; Watson et al., 1988) and its expression has been shown to be regulated by steroid hormones (Priest et al., 1995).
Expression of PPE, the precursor of the neuropeptide enkephalin, is also directly correlated with female sexual behavior. In VMH and MPO of control animals the level of PPE mRNA exhibited no sex difference, but marked changes were caused by the two treatments. PPE mRNA levels in male and female VMH almost doubled after

exposure to the lower PBDE99 dose, whereas the PCB mixture caused the opposite effect, a pronounced down-regulation.
However, the contrasting effect of the two types of chemicals in one of the regions (VMH) and the regional differences indicate that the underlying mechanisms are complex also in the case of PPE. The marked difference between low and high PBDE dose in VMH is interesting. It might be part of a bell-shaped dose-response curve, but this could only be decided with an extended dose range.

Acute estrogen challenge

Developmental alterations of regulatory systems need not become manifest under steady state conditions, when certain deficits may be balanced. Also, steady state mRNA levels are difficult to interpret in chronic exposure situations. More direct informations on the functional state of the system can be expected from challenge experiments.
A number of studies reported that in both of the examined sexually dimorphic brain regions, the expression of certain genes is regulated by estradiol in adult animals (MacLusky and McEwen, 1980; Romano et al., 1989, Lauber et al., 1991). For the assessment of PBDE99´s potential to harm this regulatory system in early stages of development, the sensitivity of target genes to acute injection of estradiol has been analyzed in adult rat offspring. In order to eliminate endogenous sex hormone production, the animals were gonadectomized two weeks prior to the experiments. The animals were given one single dose of estradiol or just the solvent as a control, and sacrificed six hours later for further analyses of the tissues.

Sensitivity of PR mRNA expression in VMH and MPO of developmentally exposed adult offspring

After administration of a single dose of estradiol PR mRNA was significantly induced in VMH and MPO of adult gonadectomized control animals as compared to the respective vehicle injection. Females seemed to be more sensitive than males by exhibiting a higher relative increase in PR mRNA than males. In former studies it has been shown that there is in fact a quantitative sex difference in the induction of PR mRNA by estrogens (Lauber et al., 1991). The observations on controls within the study at hand are also supported by earlier findings of higher levels of estrogen-

inducible PR mRNA in specific nuclei of the female hypothalamus (Rainbow et al., 1982; Brown et al., 1987 and 1990). As to the other examined region, the medial preoptic area (MPO), the present study did not show any sex difference in the reaction of PR mRNA levels to estradiol administration. Both control groups exhibited similar induction patterns of PR mRNA. This is in keeping with earlier findings of Brown and collaborators who in 1987 found no differences in the reaction pattern between the two sexes.

The PR mRNA levels of vehicle-injected rats, which reflect the “baseline level” of gonadectomized offspring, exhibited small differences. These changes are difficult to interpret, since the molecular actions of PBDE99 are not yet well characterized, and Aroclor represents a mixture of PCB congeners. The slight increase observed in the majority of groups could possibly result from weak estrogenic actions (Meerts et al., 2001).

However, we are more interested in the magnitude of PR mRNA induction relative to the vehicle-injected level, since this parameter reflects the sensitivity to estrogen.

Prenatal treatment with PBDE 99 or PCB changed the sensitivity of males severely. PR mRNA induction by estradiol was generally reduced in VMH as well as MPO of both sexes. The only exception is male VMH, which exhibited enhanced PR mRNA induction in the group exposed to the higher PBDE99 dose, but reduced induction in the lower dose PBDE99 group.

Prenatal PBDE 99 as well as PCB exposure affected the sensitivity of PR expression to estradiol in a rather dramatic way. In male VMH and male and female MPO, the response to estradiol was virtually abolished, PR mRNA remained at 100% of the vehicle-injected group. As in the case of the data collected under steady state conditions, it cannot be definitely decided whether the changes in responsiveness to estradiol are the result of interactions with developmental mechanisms, or of actions of the chemicals still present in the adult offspring. If PBDE99 could be regarded as a weak partial agonist at ER, then the reduced induction of PR mRNA by estradiol might tentatively be explained by antagonistic actions. However, this would not explain the opposite effect – enhanced induction – in VMH of PBDE99 10mg/kg-exposed males. Also, it should be noted that PCB have been shown to change key elements of endocrine regulation such as aromatase activity in neonatal brain (Hany

et al., 1999). Thus it appears that either test-compound had a high developmental impact on this delicately balanced regulatory system.

The changes in sensitivity strengthen the findings of the baseline experiment of the present study where prenatal treatment with PBDE99 provoked changes in PR mRNA levels, leading to a loss of sexual dimorphism in the VMH.

Sensitivity of PPE mRNA expression in VMH and MPO of developmentally exposed adult offspring

As a second target gene to analyze influences of developmental exposure to PBDE99 on the sensitivity to estradiol, preproenkephalin (PPE) was chosen. Since a number of studies already have shown that PPE gene expression is inducible by administration of estrogens in hypothalamus and medial preoptic area of gonadectomized rats (Romano et al., 1988, 1990; Priest et al., 1995; Segarra et al., 1998) PPE appeared to be a suitable target for this purpose.
The situation in the study at hand is rather complex with regard to this particular gene. What strikes most is the huge inter-individual variability among all treatment groups. This led to very high standard deviations, rendering statistically solid statements difficult. In the VMH of female as well as male controls there was almost no effect of estradiol on the mRNA level of PPE detectable. In contrast, previous studies reported PPE mRNA expression to increase after estradiol administration (Romano et al., 1988, 1990; Zhu et al., 2001). However, the reaction of PPE mRNA synthesis seems to be very complex, as Priest and co-workers (1995) found that in ovarectomized Long-Evans rats, treatment with estradiol leads to a multiphasic change in PPE mRNA level over the course of 48 hours, with an early peak followed by a decrease at around 4 hours and a late increase. It is difficult to asses to which time point of Priest´s study corresponds the time point of 6 hours chosen in the present study.
However, it appears that the animals were investigated during the secondary phase of decreasing PPE mRNA levels, since in untreated controls PPE mRNA was reduced by estradiol to 50-80% of the level of vehicle-injected rats in VMH. Developmental PBDE99 exposure further enhanced the repressive effect of estradiol in VMH, whereas the PCB mixture reduced the estradiol effect.

In a study carried out by Lauber and collaborators (1990) the administration of estradiol to ovarectomized female rats via incorporated capsules had no effect on PPE mRNA in the MPO. In line with that is the finding of this study with no significant effect of estradiol injection on the PPE mRNA level in MPO. Also prenatal treatment with PBDE99 or PCB had no effect in this region in females.

In the MPO of control males treatment with estradiol caused a significant suppression of PPE mRNA. This suppressive effect remained unchanged after prenatal treatment with the low dose of PBDE99 (P1-group), whereas in the high dose group of PBDE99 (P10-group) as well as the PCB treated group (Aro10), the effect of estradiol was blunted (Aro10) or even reversed (P10).

Taken together, those findings demonstrate that developmental exposure to PBDE 99 and PCB is able to influence PPE mRNA expression under steady state conditions as well as the response to acute estrogen administration, in a sex- and region-specific manner. The contrasting differences in the effect of PBDE99 on estradiol-induced changes in PR mRNA and PPE mRNA expression (reduced versus enhanced sensitivity) and the region- and sex-specific differences appear to speak in favor of changes resulting from developmental actions. In particular the combination of reduction of responsiveness to estradiol in one gene and enhanced responsiveness in another gene would be difficult to explain by a simple mechanism such as partial agonist of persisting PBDE99.

Whether the effects on PPE have any consequences on functional levels other than mRNA or whether other physiological or behavioral endpoints are affected, is not known. Lauber and coworkers (1990) have been able to show that an estrogen induced increase of PPE mRNA levels in the VMH correlates directly with the induction of lordosis behavior in a dose dependent manner. Also mRNA levels of PPE are directly correlated with the female estrous cycle (Funabashi et al. 1995). In addition it was demonstrated that blockage of PPE expression by injection of antisense oligos for PPE mRNA into the hypothalamus results in a rather dramatic repression of female lordosis behavior (Nicot et al., 1997), similar to the effect of PR mRNA antisense oligos.

Adult Reproductive Functions

Estrous cycle

The female estrous cycle was severely affected by both treatments, PCB as well as PBDE99 in all treatment groups. Cycles were studied continuously by taking vaginal smears over a period of about two to three weeks. The alterations of the estrous cycle in the treated females suggest actions of an endocrine disrupter.

Sexual behaviour

According to Zipse (2000) the proceptive behavior of female rats can be altered after animals have been treated with sex hormones. In line with this is the finding of this study with the drop down of jumping and wiggling behavior in the PBDE99-treated group as compared to the control group. This may have been caused by the potential action of PBDE99 as an endocrine disrupter.

It seems plausible that with the PBDE99-exposed females showing less proceptive behavior and thus the male being less attracted, the number of mounts is significantly decreasing. In addition, we observed a dramatic decrease of the lordosis quotient, which represents the quotient of the total numbers of lordosis divided by the total numbers of mounts. This indicates that female sexual behavior of PBDE99-exposed offspring was also impaired, besides the alteration of proceptive behavior. What could not be clarified within this study is the mechanism underlying the disturbed female sexual behavior. It might have been linked in part to the irregular estrous cycle. Both phenomena may have been caused by alterations at brain level as indicated by our molecular biology data. Since female sexual behavior depends on the estrous cycle, females were always taken in proestrous/estrus for the behavioral experiments. However, due to their disturbed estrous cycle it was not easy to determine the exact estrous cycle state of the PBDE99-treated females. Therefore altered behavior in the experiment may just reflect the changed estrous cycle after treatment with PBDE99.

Conclusions

Possible interactions of environmental chemicals with the neuro-endocrine system are a matter of serious concern. Being involved in reproduction, impaired function might threaten fitness and survival of species. Yet, so far the impact of endocrine active environmental chemicals on the control of central neuroendocrine processes has not earned as much interest in research as its possible consequences urgently would suggest. Instead the focus has been mainly on reproductive organs. Merely organochlorine pesticides and PCBs have been shown to interfere with processes of sexual brain differentiation (vom Saal et al., 1995; Hany et al., 1999). Alterations in gene expression patterns following developmental exposure to a chemical UV-filter, 4-methylbenzylidene camphor (4-MBC), have recently been examined in sexually dimorphic brain regions as well as in reproductive organs (Maerkel et al., 2005).
The present study could show that developmental exposure to PBDE 99, an endocrine active flame retardant present in many environmental samples (Zennegg et al., 2002), can affect processes of sexual brain differentiation on molecular integration levels. These effects were manifest in a range of PBDE 99 dosages that did not show any signs of general toxicity, as indicated by the absence of effects on survival rate and body weight.
However, the significance of changes in mRNA levels following developmental exposure to PBDE is difficult to asses in terms of possible consequences on higher integration levels. More information on the functional state of regulatory neuroendocrine systems was obtained from the estrogen challenge experiment. It was designed to assess the efficiency of transcriptional effects of estrogens (i. e. estradiol) on the induction or repression of target genes. The data obtained suggest severe changes in the sensitivity of genes involved in neuroendocrine control to natural estrogens.
However, the changes at the molecular level might as well reflect processes of adaptation to newly arising environmental conditions and a new chemical milieu under which the organisms have to develop. The assessment of a possible actual threat to species preservation has to be carried out at higher biological integration levels. The behavioral study performed in this project was intended to provide first insight into that matter.
As already mentioned, the data on PR mRNA expression in VMH suggested possible effects on female sexual behavior, induction of progesterone receptor in VMH by

estrogen is central to the induction of female sexual behavior. The massive reduction of the lordosis reaction (lordosis quotient) in females exposed to the higher dose of PBDE99 (the only dose tested), may have been linked with the reduction of progesterone receptor gene expression in the VMH.
The alterations in estrogen target gene regulation at brain level may also have played a role in the severely altered estrous cycle. This may in turn have influenced behavior. It turned out to be very difficult in the PBDE-treated group to determine the actual cycle state, because the vaginal smears showed different cell types at one time. It was necessary for this group to follow the cycle for some time and then select a state that in terms of the proportion of cell-types came closest to the state required for the behavioral experiment (i. e. proestrous/estrous). Alterations in estrous cycle and sexual behavior probably result both, at least in part from a disturbed differentiation of the CNS during early stages of development.
The present study illustrates that, in addition to classical toxicological parameters, the analysis of gene expression can be considered as a novel approach to study effects of endocrine active chemicals on brain differentiation.

Outlook

In order to asses the practical threat of endocrine disrupting chemicals such as PBDE99 to the biological fitness of animals, it would be interesting to find out more about the impact on reproduction. Therefore further experiments would be useful which show, whether developmentally exposed F1 females would actually get pregnant and if their maternal behavior is affected in a way that would make them incapable of raising offspring.

“Save the best for last”
From October 2004 on penta- and octa-bromodiphenyl ether are prohibited in the European Union (directive 2003/11/EC, published in the “Official Journal of the European Union”, February, 15th 2003). “In order to protect health and the environment the placing on the market and the use of pentaBDE and octaBDE and the placing on the market of articles containing one or both of these substances should be prohibited.”

ABBREVIATIONS

Aro: Aroclor 1254

BDE: brominated diphenyl ethers

ERα: Estrogen Receptor α

ERβ: Estrogen Receptor β

GD: gestational day

MPO: medial preoptic region

PR: Progesteron Receptor

PBDEs: polybrominated diphenyl ethers

PBDE47: 2,2',4,4'-tetrabromodiphenyl ether

PBDE99: 2,2',4,4',5-pentabromodiphenyl ether

PN: postnatal day

PPE: preproenkephalin

P1: PBDE 99 1mg/kg

P10: PBDE 99 10mg/kg

VMH: ventromedial hypothalamus

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PUBLICATIONS

Articles

Lichtensteiger, W., Ceccatelli, R., Conscience, M., Cotton, B., Durrer, S., Faass, O., Fleischmann, I., Ma, R., Maerkel, K., Schlumpf, M. (2002). Newly arising endocrine disruptors: UV screens and PBDE. Reprod. Toxicol. 16: 397-398

Lichtensteiger, W., Ceccatelli, R., Faass, O., Ma, R., and Schlumpf, M. (2003). Effects of polybrominated diphenylether and PCB on the development of the brain-gonadal axis and gene expression in rats. Organohalogen Compounds, Vol. 61:84-87

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Abstracts

R. Ceccatelli, O. Faass, I. Fleischmann, M. Conscience, M. Schlumpf and W. Lichtensteiger. Development in rat after prenatal exposure to the flame retardant PBDE99. USGEB congress 2002 in Lugano, Switzerland

O. Faass, R. Ceccatelli, I. Fleischmann, M. Schlumpf and W. Lichtensteiger. Neuroendocrine development in rat after prenatal exposure to flame retardant PBDE99 or to PCBs. USGEB congress 2003 in Davos, Switzerland

W. Lichtensteiger, R. Ceccatelli, O. Faass, I. Fleischmann, R. Ma and M. Schlumpf. Effects of Polybrominated Diphenylether (PBDE) and PCB on Reproductive Organ and Brain Development and Gene Expression. SETAC conference 2003, Hamburg, Germany

O. Faass, R. Ceccatelli, I. Fleischmann, M. Schlumpf and W. Lichtensteiger. Effects of developmental exposure to PBDE or PCB on gene expression in rat brain. USGEB congress 2004 in Fribourg, Switzerland

O. Faass, R. Ceccatelli, I. Fleischmann, M. Schlumpf and W. Lichtensteiger. Female sexual behavior and estrogen target gene regulation in rat brain regions After developmental exposure to the flame retardant PBDE99. USGEB congress 2005 in Zurich, Switzerland

O. Faass, R. Ceccatelli, I. Fleischmann, M. Schlumpf and W. Lichtensteiger. Female sexual behavior and estrogen target gene regulation in rat brain regions After developmental exposure to the flame retardant PBDE99. SETAC conference 2005 in Basel, Switzerland

O. Faass, K. Maerkel, S. Durrer, M. Henseler, W. Lichtensteiger and M. Schlumpf. Female sexual Behavior and Estrogen Target Gene Regulation in Rat Brain sex dimorphic Regions following developmental Exposure to the UV Filters 4-MBC and 3-BC and the Flame Retardant PBDE 99. NRP50 Annual Meeting 2005, Cadro, Switzerland

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